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(54) Title: PROTEIN SIGNATURE ANALYSIS

(57) Abstract

A protein signature analysis is obtained using a peptide ladder library. The molecular signature of a protein is defined to be that subsequence of amino acid positions within the protein which are essential for the protein to bind to a target molecule. The molecular signature may be determined by screening a peptide ladder library which corresponds to the protein against the target molecule. The peptide ladder library is a library of m peptides wherein each peptide has an amino acid sequence of length m corresponding to an amino acid sequence of the protein, with one exception, viz. peptide$_m$ has a substitute amino acid at position$_m$ and the substitute amino acid is attached by a labile bond to its neighboring amino acid. Screening the peptide ladder library against the target molecule results in a division of the original mixture into a positive (functional) pool and a negative (non-functional) pool. The pools are separated and subjected to cleavage to obtain cleavage products. Analysis of the cleavage products by mass spectrometry identifies the positions that are essential for the protein to bind to its molecular target.

Parent peptide sequence: PFKK-[GDILRIRDKP]-EEAcpRLKLKAR

Target Peptide Array

PFKK	▨	I	L	R	I	R	D	K	P	EEAcpRLKLKAR
PFKK	G	▨	L	R	I	R	D	K	P	EEAcpRLKLKAR
PFKK	G	D	▨	R	I	R	D	K	P	EEAcpRLKLKAR
PFKK	G	D	I	▨	I	R	D	K	P	EEAcpRLKLKAR
PFKK	G	D	I	L	▨	R	D	K	P	EEAcpRLKLKAR
PFKK	G	D	I	L	R	▨	D	K	P	EEAcpRLKLKAR
PFKK	G	D	I	L	R	I	▨	K	P	EEAcpRLKLKAR
PFKK	G	D	I	L	R	I	R	▨	P	EEAcpRLKLKAR
PFKK	G	D	I	L	R	I	R	D	▨	EEAcpRLKLKAR

▨ = Gly-SβAla

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PROTEIN SIGNATURE ANALYSIS

Description

Technical Field of the Invention:

The present invention relates to methods for analyzing, altering, and controling the structural basis for protein binding to target molecules. More particularly, the present invention is directed to peptide ladder libraries corresponding to a protein, protein fragment, or other bioactive peptide and to the use of peptide ladder libraries for obtaining a protein signature analysis.

Background of the Invention:

One of the major strategies for determining the relationship between the chemical structure of a peptide and its biological activity is to systematically alter the covalent structure and observe the effect on function. Through the use of chemical synthesis, a wide variety of modifications can be made. For example, •N-methylation and the use of ester bonds can probe backbone interactions (Arad et al. *Biopolymers* 1990, *29*, 1633-1649; Bramson et

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al. *J. Biol.Chem.*1985, *260*, 15452-15457; Caporale et
al. In: *Peptides: Structure and Function, Proceedings
of the Tenth American Peptide Symposium;* Marshall,
G.F. Ed. Escom:Leiden: The Netherlands, 1988, pp.

5 449-451), while sidechain contributions can be probed
using D-amino acid or Alanine/Glycine substitutions
(Konishi et al. In: *Peptides: Structure and Function,
Proceedings of the Tenth American Peptide
Symposium,*Marshall, G.F. Ed. Escom, Leiden: The

10 Netherlands, 1988, pp. 479-481; Tam et al. In
*Peptides:Proceedings of the Eleventh American Peptide
Symposium;* Rivier, J. E.; Marshall, G. R. Ed.;
Escom: Leiden, The Netherlands, 1990. pp 75-77). As
traditionally practiced, a separate analogue must be

15 prepared and assayed for each position in the peptide
sequence that is to be studied.

An alternative, currently popular method of
studying peptides is through combinatorial chemistry.
This approach has had a major impact on the study of

20 the molecular basis of peptide activity and has
contributed to the search for new biologically active
peptides (Thompson et al. *Chem. Rev.* 1996, *96*, 555-
600; Gordon et al. *J. Med. Chem.* 1994, *37*, 1385-
1401; Scott et al. *Curr. Op. Biotech* 1994, *5*, 40-48)

25 'Multiple Peptide Synthesis' has extended the
traditional approach by allowing peptides to be
synthesized simultaneously (Geysen et al. *J. Proc.
Natl. Acad. Sci. USA* 1984, *81*, 3998-4001; Houghten et
al. *Proc Natl. Acad. Sci. USA* 1985, *82*, 5131-5134).

30 The individual peptide products are spatially
separated and can be analyzed either attached to a

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solid support or in solution. Established 'split
synthesis' (Furka et al. *Int. J. Pept. Prot. Res.*
1991, 37, 487-494; Lam et al. *Nature* **1991**, *354*, 82-
84) procedures allow for the rapid generation of huge
5   numbers of peptide sequences through the repetition
of a simple divide, couple and recombine process.
The compositional diversity made possible by this
approach is advantageous for the discovery of new
'lead' compounds since, in principle, all possible
10   structural variants can be explored for the desired
activity and only the few active oligomers of
interest need to be individually identified (Furka et
al. *Int. J. Pept. Prot. Res.* **1991**, 37, 487-494; Lam
et al. *Nature* **1991**, *354*, 82-84). However, where
15   information about a complete set of functional and
non-functional components is desired over many
positions in a peptide sequence, such libraries are
too complex to fully characterize and may have
limited utility.
20   A more systematic investigation of the molecular
basis of peptide function requires a different type
of molecular diversity. Instead of a peptide mixture
of high compositional diversity, it would be useful
to construct an array of peptides which differ from
25   each other in a precise and defined manner. In
principle, one way to access this population would be
as a minor fraction of a large, fully combinatorial
library. For example, such an array of analogues
could consist of all peptides which differ from a
30   target sequence by a single amino acid substitution
at each position in a peptide sequence (cf. 'Ala
scans'). By removing this defined subset of

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analogues from the context of a complex, fully
combinatorial mixture of peptides, handling and
analysis would be greatly simplified and a more
useful profile of the effects of substituting the
5   amino acid throughout the peptide chain would be
obtained.   Current split resin methods do not allow
for this type of control over the composition of a
peptide library.   (Furka et al. *Int. J. Pept. Prot.
Res.* 1991, 37, 487-494; Lam et al. *Nature* 1991, *354,*
10   82-84).

Typically, to investigate the molecular basis of
protein function systematic modifications are made to
the protein structure and the effects of those
modifications on the properties of the protein are
15   evaluated.   Site-directed mutagenesis (Smith et al.
*Angew. Chem. Int. Ed. Engl.*1994, *33,* 1214-1220) has
been the principle tool used to implement this
approach and has given many insights into the
contribution of individual sidechains to protein
20   function.   In particular, 'alanine scanning' (Wells
et al. *Methods in Enzymology* 1991, *202,* 390-411) has
been used to identify specific amino acid sidechains
involved in ligand binding interactions.   This
technique involves the sequential substitution of
25   native amino acids by individual alanine residues
which are regarded as functionally and structurally
neutral.   To extend the repertoire of modifications
beyond the twenty genetically encoded amino acids,
methods have been developed to substitute non-natural
30   groups into proteins (Noren et al. *Science.* 1989.
244, 182-185).   Although a variety of both novel

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sidechain and backbone modified proteins have been
generated, there are apparent limits to the
modifications possible using the methods of molecular
biology and ribosomal synthesis (Ellman et al.
5   *Science* **1991**, *255*, 197-200; Cornish et al. *Angew Chem
Int. Ed. Engl.* **1995**, *34*, 621-633).

Recent advances in the total synthesis of
polypeptides have opened the world of proteins to
direct application of the tools of organic chemistry
10   (Schnölzer et al. *Science* **1992**, *256*, 221-225; Jackson
et al. *Science* **1994**, *266*, 243-247; Dawson et al
Science **1994**, *266*, 776-779; Canne et al. *J. Am. Chem.
Soc.* **1995**, *117*, 2998-3007; Liu et al *J. Am. Chem.
Soc.* **1995**. *118*, 307-312; Englebretsen et al. *Tet.
15   Lett.* **1995**, *36*, 8871-8874). Using total chemical
synthesis, a variety of protein analogues has been
synthesized. Of particular note have been proteins
containing ß-turn mimics (Baca et al. *Prot. Sci.*
1993, *2*, 1085-1091), N-methylated amino acids
20   (Rajarathnam et al. *Science* **1994**, *264*, 90-92),
modified backbone atoms (Baca et al *J. Am. Chem. Soc.*
1995, *117*, 1881-1887), and mirror image proteins
composed entirely of D-amino acids (Zawadzke et al.
J. Am. Chem. Soc. **1992**, *114*, 4002-4003; Milton et al.
25   *Science* **1992**, *256*, 1445-1448; Fitzgerald et al. *J.
Am. Chem. Soc.* **1995**, *117*, 11075-11080; Schumaacher et
al. *Science* **1996**, *271*, 1854-1857). In addition,
important insights into the mechanism of action of
enzymes have been attained through the total chemical
30   synthesis of unique analogues (Baca et al. *Proc.
Natl. Acad. Sci. U.S.A.* **1993**, *90*, 11638-11642).

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Although structure-function relationships in proteins can be studied using individual analogues prepared by either recombinant or chemical techniques, development of a profile of effects across the whole protein molecule is hindered by the time and effort required to generate and analyze multiple protein analogues (Matthews et al. *Ann. Rev. Biochem.*1993, *62*, 139-160). The use of combinatorial oligonucleotide synthesis in conjunction with protein expression in bacteria (Reidhaar-Olsen et al. *Science* 1988, *241*, 53-57; Gregoret et al. *Proc. Natl. Acad. Sci. USA*. 1993. *90*. 4246-4250) or on phage (Scott et al. *Science* 1990, *249*, 386-390; Lowman, H. B. Bass, S.H.; Simpson, N.; Wells, J. A. *Biochemistry* 1991. *30* 10832-10838) has provided a powerful method for studying large numbers of analogue proteins. These techniques allow pools of expressed proteins to be probed for a desired function. With appropriate screening procedures, a statistical sampling of numerous functional protein variants can be analyzed and identified (Gu et al. *Protetin Science* 1995, *4*, 1108-1117). This strategy has proved to be powerful for generating variant proteins with new or optimized functions (Lowman et al. *J. Moll. Biol.* 1993, *234*, 564-578; Rebar et al. *Science* 1994, *263*, 671-673). However, studies designed to elucidate the molecular basis of protein function have been complicated by the necessarily incomplete characterization of the numerous protein analogues generated, and also by limitation to the naturally encoded amino acids.

In applying molecular diversity to the study

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protein function it would be useful to combine the
valuable information gained by systematic
modification through chemical synthesis with the
advantages of combinatorial methods.

5   What is needed is an integrated approach to the
preparation of a defined array of peptide and protein
analogues in a single synthesis, their functional
separation into active and inactive pools, and a
simple one step readout of the composition of the

10   self-encoded mixtures.

Summary of the Invention:

There are three aspects to the invention:

1.   A combinatorial method for synthesizing a
                peptide ladder library corresponding to a
15   protein, protein fragment, or other
                bioactive peptide.

2.   A method for screening the peptide ladder
                library with respect to a binding function.

3.   A method for identifying active or inactive
20   components of the peptide ladder library,
                i.e. identification of a protein signature
                for the protein or protein fragment under
                investigation with respect to the function
                being probed.

25   A combinatorial synthetic method for making a
peptide ladder library is illustrated in Figure 1.
The peptide ladder library is a one pot collection of

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"n" peptides, each peptide being identical to the
others in the library with respect to molarity and
structure except for the substitution of a marker at
position "n". The marker introduces a labile bond
5   into the peptide backbone, e.g. a thioester bond,
which can be selectively cleaved without cleaving
other bonds within the peptide backbone. The marker
also serves to introduce a ladder of stearic
perturbations into the peptide backbone and/or to
10   introduce a ladder of peptide side chain
substitutions. The synthetic protocol employs a
split synthesis method.

Conventional screening methods may be employed
on the peptide ladder library to separate active
15   components from inactive components within the
library. An exemplary screening protocol is
illustrated in Figure 2.

After the screening is complete, the isolated
components are analyzed as illustrated in Figure 3 to
20   obtain a molecular signature for the protein.
Briefly, the isolated components are cleaved at their
marker and analyzed. Mass spectrometry is the
preferred method of analysis. However, alternative
analytical methods include nmr (with deuterium
25   exchange), ir, and FACS. Comparison of the analysis,
e.g., ms, of the isolate with the control, i.e., an
aliquot of the entire library, provides a molecular
signature which identifies sites within the protein
responsive or unresponsive to the screening method.
30   For example, sites within the protein essential for

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binding or folding may be identified.   The protein
signature of the Crk-N/C3G interaction is illustrated
in Figure 3.

5   Successive iterations of the method of the
invention can be employed to obtain a complete
deconstructive analysis of a protein, even if the
structure of the protein is unknown. The invention
may be employed to characterize protein interactions
and can facilitate the design of new therapeutics
10   which are dependent upon such protein interaction.

          One aspect of the invention is directed to a
method for obtaining a molecular signature of a
protein.   The protein is of a type which has an amino
acid sequence with length m, each amino acid position
15   being represented by $(aa)_n$ where $1 \leq n \leq m$.   The protein
is also of a type which has a binding affinity with
respect to a target molecule under binding
conditions. The molecular signature then defined by a
subsequence of the amino acid sequence of the
20   protein.   The subsequence is selected from amongst
those positions $(aa)_n$ of the protein which, if
individually replaced by a substitute amino acid,
lead to a loss of binding affinity by the protein
with respect to the target molecule.

25   The method employs a peptide ladder library.
The peptide ladder library has m peptides.   Each of
the peptides is represented by $(peptide)_n$, where
$1 \leq n \leq m$.   Each peptide has the same amino acid sequence
as the protein except that position $(aa)_n$ of

(peptide)$_n$ is replaced by a substitute amino acid.
Preferred substitute amino acids include alanine and
glycine. If only one substitute amino acid is
employed, then the peptide has a footprint the size

5   of one amino acid. In alternative embodiments, the
footprint may include two or three substitute amino
acids. The substitute amino acid at position (aa)$_n$
is linked to the amino acid at position (aa)$_{n+1}$ by
means of a labile bond. Preferred labile bonds are

10   thioester bonds and ester bonds.

The peptide ladder library is then contacted
with the target molecule under binding conditions in
order to form bound peptides and unbound peptides.
The bound peptides are bound to the target molecule;

15   the unbound peptides are not. The unbound peptides
are then separated from the bound peptides from said
Step B in order to obtain separated unbound peptides.
Each of the separated unbound peptide has the
substitute amino acid only at position (aa)$_n$ which

20   constitute the subsequence that define the molecular
signature of the protein with respect to the target
molecule. The labile bond of the separated unbound
peptides are then cleaved in order to produce peptide
cleavage products. Each peptide cleave product

25   corresponds to one of the positions (aa)$_n$ from the
subsequence which defines the molecular signature.
The subsequence which defines the molecular signature
of the protein is then constructed using the identity
of the peptide cleavage products to identify the

30   subsequence of amino acid positions that are
essential for binding to the target molecule.

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Alternative substitute amino acids include the
following: L-alanine, L-arginine, L-aspartic acid, L-
asparagine, L-cysteine, L-cystine, L-glutamic acid,
L-glutamine, L-glycine, L-histidine, L-isoleucine, L-
5   leucine, L-lysine, L-methionine, L-phenylalanine, L-
proline, L-serine, L-threonine, L-tryptophan, L-
tyrosine, L-valine, D-alanine, D-arginine, D-aspartic
acid, D-asparagine, D-cysteine, D-cystine, D-
glutamic acid, D-glutamine, D-glycine, D-histidine,
10   D-isoleucine, D-leucine, D-lysine, D-methionine, D-
phenylalanine, D-proline, D-serine, D-threonine, D-
tryptophan, D-tyrosine, D-valine, L-α-aminobutyric
acid, D-α-aminobutyric acid, L-γ-aminobutyric acid,
D-γ-aminobutyric acid, L-e-aminocaproic acid, D-
15   e-aminocaproic acid, L-homophenylalanine, D-
homophenylalanine, L-alloisoleucine, D-
alloisoleucine, L-β-2-napthylalanine, D-β-2-
napthylalanine, L-norvaline, D-norvaline, L-
ornithine, D-ornithine, L-pyridyl alanine, D-pyridyl
20   alanine, L-2-thienylalanine, D-2-thienylalanine L-
methyltyrosine, D-methyltyrosine, L-citrulline, D-
citrulline, L-homocitrulline, and D-homocitrulline.

In an alternative mode, the molecular signature
of the protein is determined as described above
25   except that the analysis is performed on the bound
peptides are separated from the unbound peptides.
Each of the separated bound peptides lacks any
substitute amino acid at position $(aa)_n$ from the
subsequence which defines the molecular signature of
30   the protein. The labile bonds of the separated bound
peptides are then cleaved to form peptide cleavage

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products.   Each peptide cleave product corresponds to
one of the positions $(aa)_n$ not included within the
subsequence which defines the molecular signature.
Accordingly, in this mode of the invention, after
detecting and identifying each of the peptide
cleavage products, the subsequence which defines the
molecular signature of the protein with respect to
the target molecule is constructed by identifying
amino acid positions $(aa)_n$ which doe not correspond
to any of the peptide cleavage products.

Another aspect of the invention is directed a
peptide ladder library corresponding to a protein.
The protein is of a type which has a binding affinity
with respect to a target molecule under binding
conditions.   The protein is also of a type which has
an amino acid sequence with length m where $1 \leq n \leq m$.
Each amino acid position within the protein is
represented by $(aa)_n$.   The peptide ladder library
then comprises m peptides, each peptide being
represented by $(peptide)_n$, where $1 \leq n \leq m$.   Each peptide
within the library has the same amino acid sequence
as the protein except that position $(aa)_n$ of
$(peptide)_n$ is replaced by a substitute amino acid.
The substitute amino acid at position $(aa)_n$ is
linked to the amino acid at position $(aa)_{n+1}$ by means
of a labile bond.   If only one substitute amino acid
is employed, then the peptide has a footprint the
size of one amino acid.   In alternative embodiments,
the footprint may include two or three substitute
amino acids.   Preferred labile bonds include
thioesters and esters.   Preferred substitute amino

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acids are alanine and glycine.

Another aspect of the invention is directed to a
method for constructing a peptide ladder library
corresponding to a protein. The protein is of a type
5   which has an amino acid sequence with length m. Each
amino acid position of the protein may be represented
by $(aa)_n$ where $1 \leq n \leq m$. The peptide library includes m
peptides. Each peptide may be represented by
$(peptide)_n$, where $1 \leq n \leq m$. Each peptide has the same
10 amino acid sequence as the protein except that
position $(aa)_n$ of $(peptide)_n$ is replaced by a
substitute amino acid. The substitute amino acid at
position $(aa)_n$ is linked to the amino acid at
position $(aa)_{n+1}$ by means of a labile bond. A first
15 reaction vessel may be provided which contains a
first pool of nascent peptides having a length of m-
n. The amino acid sequence of the nascent peptides
runs between n+1 and m of the protein. The nascent
peptides are attached to a matrix material. A second
20 reaction vessel may be provided which contains a
first pool of nascent ladder peptides having a
length of m-n. The amino acid sequence runs between
n+1 and m of the protein except that each (nascent
ladder peptide)$_p$ has the substitute amino acid at
25 position $(aa)_p$, where $n+1 \leq p \leq m$. The nascent ladder
peptides are attached to a matrix material. An
aliquot of matrix material is then transferred from
the first reaction vessel to a third reaction
vessel. Elongation reactions are then performed in
30 each of the three reaction vessels. The first pool
of nascent peptides in the first reaction vessel is

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elongated by addition of the amino acid of position $(aa)_n$ to form a second pool of nascent peptides having a length of m-n+1; the aliquot of nascent peptides in the third reaction vessel is then elongated by addition of the substitute amino acid of position $(aa)_n$ by means of labile bond to form a nascent ladder $(peptide)_n$ having a length of m-n+1; and the first pool of nascent peptide ladders in the third reaction vessel is elongated by addition of the amino acid of position $(aa)_n$ to form a partial second pool of nascent peptide ladders having a length of m-n+1. After the elongation reactions are complete, the product of the third reaction vessel is transferred to the second reaction vessel to complete the second pool of nascent peptide ladders having a length of m-n+1. The above process may then be repeated until n=1 and the second reaction vessel contains the sought after peptide ladder library.

Description of Figures:

Figure 1 represents the solid-phase peptide synthesis strategy used to scan a synthetic marker (dark oval) through sequential dipeptide units in a polypeptide sequence. Each member of the resulting peptide family contains a single copy of the marker at a unique dipeptide site. By splitting and subsequently recombining the peptide-resin, all the members of the polypeptide family can be generated in a single synthesis. A modified solid phase peptide synthesis

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methodology has been developed that makes it possible
to prepare all members of the array of protein
analogues concurrently in the course of a single
synthesis. This simple procedure involves the use of

5   two reaction vessels. At each stage of the synthesis
a small aliquot of the peptide-resin is removed from
the first vessel, and the analogue moiety attached to
the growing peptide chain. The resin aliquot is then
transferred to the second reaction vessel and the

10   remainder of the amino acids in the sequence are
coupled. Continual siphoning of resin aliquots from
vessel #1 into vessel #2 (with analogue attachment in
between), results in the generation of the complete
protein array as a single product mixture. Use of

15   this split-resin procedure ensures that each
component of the array contains only a single copy of
the analogue at a unique and defined position. The
synthetic marker can be designed to probe the
importance to structure and function of side-chain

20   atoms, backbone atoms or both.

Figure 2 represents a one experiment cycle of
iterative signature analysis. Multiple rounds of
this cycle can be performed with the information from
previous cycles being incoporated into each

25   successive iteration.

Figure 3 illustrates preliminary iterative signature
analysis data on residues 156-165 of the peptide Crk-
N. a) Represents a comparison of the elution profiles
obtained for the protein family using a C3G-peptide

30   agarose column and a Leucine-Enkephalin agarose

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column (control). Note that purified synthetic Crk-N
binds the C33G-peptide in solution with a K_D of 2.3 μM
(recombinant Crk-N = 1.9 μM). b) Represents HPLC
profiles (all 25-45% acetonitrile over 30 minutes)
5   obtained from the high salt was (non specific) and
ammonium acetate wash (specific) for the two columns.
c) Represents the theoretical masses of peptide
fragments produced upon ammonium acetate cleavage of
a Crk-N family. Single letters refer to amino acid
10   pairs substituted with the -Gly-SCH_2CH_2CO- marker. d)
Comparison of the MALDI MS spectra of the ammonium
acetate wash and the entire 9 component synthetic
Crk-N family cleaved with ammonium acetate. In both
cases the N-terminal peptide ladder is observed at a
15   much higher intensity than the equimolar C-terminal
peptide ladder.

Figure 4 illustrates the basic strategy for the
synthesis of defined arrays of peptide analogues.
The general approach is to have two main reaction
20   vessels, one for unmodified peptide-resin, A, and the
other for modified peptide-resin, B. Standard
stepwise solid phase peptide synthesis of the parent
amino acid sequence is performed in vessels A and B.
Modifications to the sequence are made in a single
25   auxiliary vessel, 1. At the beginning of each step
in which introduction of an analogue structure is
desired, a sample of peptide-resin is transferred
from A to 1, where it is modified and then
transferred from 1 to B after completion of that
30   cycle of synthesis in both A and B.

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Figure 5 illustrates the folding step for a family of peptides.

Figure 6 illustrates the screening method of the peptide ladder library with respect to binding

5   function.

Figure 7 illustrates the readout step "unzipping the peptide" which reveals the latent chemistry.

Figure 8 illustrates an anlaysis of components using mass spectroscopy, nuclear magentic resonance, HPLC,

10   IR or FACS.

Figure 9 illustrates the process of the molecular signature analysis.

Figure 10 illustrates the design of a peptide ladder from the peptide sequence of a protein, protein

15   fragment of bioactive peptide.

Figure 11 illustrates the scanning of a marker which introduces a labile bond into the peptide backbone. The labile bond can then be selectively cleaved without claving other bonds within the peptide

20   backbone. The marker also serves to introduce a ladder of steric perturbations into the peptide backbone and/or introduce side chain substitutions.

Figure 12 illustrates a representative sample of the readout chemistry to introduce a labile bond into the

25   peptide.

Figure 13 illustrates a process which comprises the

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use of iterative steps as needed. The steps can be
generally organized as 1) sequence info. 2) scan a
perturbation 3) selection and 4) feedback.

Figure 14 illustrates an example of a peptide ladder
5   library corresponding to a protein fragment of the
SH-3 domain with the sequence -KGDILRIRDKP-.

Figure 15 illustrates: (A) the target composition of
the nine member array of peptide analogues. The
sequence PFKKGDILRIRDKPEE was derived from residues
10   152-167 of the murine cCrk SH3 domain and the C-
terminal AcpRLKLKAR sequence was used to facilitate
analysis by MALDI mass spectrometry'; (B) Synthetic
operations required for the synthesis of a peptide
array consisting of nine overlapping dipeptide
15   analogues over a ten amino acid sequence. The
synthesis was performed in a single day.

Figure 16 illustrates the analysis of a nine
component array of peptide analogues. A) Analytical
HPLC of crude full length product (gradient, 20-50%
20   buffer B over 30 minutes). B) MALDI mass spectrum of
crude full length product. Unlabelled peaks at lower
mass are termination byproducts from the synthesis.
C) Analytical HPLC of hydroxylamine-cleaved HPLC
product on the same gradient. D) MALDI readout of
25   hydroxylamine-cleaved peptide array [Peaks with * are
N-terminal-containing fragments; unlabeled peaks are
C-terminal containing fragments].

Figure 17 illustrates the analytical HPLC of the
chemical cleavage of model peptides containing labile

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backbone bonds. A) Cleavage of a thioester-
containing peptide with hydroxylamine. B) Cleavage
of an ester-containing peptide by hydrolysis.

5 Figure 18 illustrates the schematic representation of
the cleavage of the nine component array of peptide
analogues. A) Full length array of nine peptide
analogues. B) Cleaved array of peptide analogues.
The mixture consists of eighteen peptides
corresponding to nine N-terminal fragments and nine
10 C-terminal fragments.

Figure 19 illustrates the relation of the MALDI
spectrum to the peptide C-terminal fragment array.
The horizontal mass scale spectrum has been inverted
to align it with the standard N-to-C terminal
15 orientation of the peptide sequence. The peaks
corresonding to the nine C-terminal peptide fragments
are clearly resolved and can be assigned
sequentially. In addition to the position of the
peak in the mass spectrum, the mass difference
20 between adjacent peaks identifies the individual
amino acids in the peptide sequence that has been
subjected to analoguing. The starred peak
corresponds to the callibrant.

Figure 20 illustrates the principle of protein
25 signature analysis. [1] Total chemical synthesis is
used to generate an array of protein molecules
derived from a single amino acid sequence. An
analogue chemical structure (represented by the the
red-&-blue rectangles) is systematically incorporated

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at defined positions in the polypeptide chain. [2]
The array of protein analogues is subjected to
functional selection, resulting in separation into
two populations: active and inactive. [3] The

5   composition of each pool of analogues is then
determined in a single step using a chemical readout
system expressly built into the molecule for that
purpose. This provides a signature relating the
effects on function to substitution of the analogue

10   structure throughout the region of interest in the
protein molecule.

Figure 21 illustrates an integrated strategy for the
chemical synthesis, functional separation, and
analysis of a self-encoded array of protein

15   analogues. The array of protein analogues is
prepared by total chemical synthesis in a single
procedure. Each analogue unit contains a selectively
cleavable bond. Site-specific cleavage yields
fragments that identify each protein component and

20   define the position of the analogue unit within the
polypeptide chain. This decoding procedure is
applied to the parent array of analogues, and to the
active and inactive pools after separation based on
function.

25   Figure 22 illustrates chemical Structures of Analogue
Units A. Comparison of a native dipeptide unit (top)
with the structures of the Gly-[COS]-Gly (middle) and
Gly-[COS]-ßAla (bottom) analogue units used in the
present study. B. Chemical cleavage of the thioester

30   bond within the analogue unit can be carried out

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selecively under mild conditions by treatment with
hydroxylamine at neutral pH. The thioester bond is
stable to the conditions normally used to study
proteins.

5   Figure 23 illustrates a readout of the composition of
an array of analogues of the cCrk N-terminal SH3
domain. A. The array consists of nine sub
populations, each containing a single Gly-SßAla
analogue unit. The dipeptide analogue was placed in
10   consecutive positions along the polypeptide chain,
resulting in an overlapping pattern of substitution.
B. Chemical cleavage of the thioester bond in the
analogue unit results in an array of peptide
fragments that characterizes the composition of the
15   mixture of parent protein analogues. C. The array
of peptide fragments can be read out in one step by
matrix assisted laser desorption time of flight mass
spectrometry (MALDI-TOF). The resulting pattern of
data is illustrated here for the C-terminal
20   containing family of fragments.

Figure 24 illustrates a combinatorial Readout of
Protein Analogue Arrays. Building a latent chemical
cleavage site into the analogue unit (rectangle)
means that each protein in the array will contain
25   this chemical marker at a unique position in the
polypeptide sequence. Chemical cleavage specifically
at the analogue unit gives rise to characteristic
peptide fragments, each with a unique mass indicative
of the position of the anlogue unit within the
30   sequence of the original protein analogue. Each

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protein analogue in an array is thus _self-encoded_.
Readout of these decoded peptide fragments can then
be performed, in one operation, using MALDI mass
spectrometry.

5   Figure 25 illustrates an application of protein
signature analysis to a twenty residue region of the
N-terminal SH3 domain of murine c-Crk. A.   The
highlighted amino acid sequence (residues 146-165)
was substituted by the dipeptide analogue Gly-[COS]-
10   ßAla, giving a 19-member array of synthetic analogue
proteins. B. Signature obtained for the parent array,
after cleavage with neutral hydroxylamine and
analysis by MALDI mass spectrometry. Only the family
of N-terminal fragments was observed in the spectrum.
15   The C-terminal fragments, although necessarily
present, are not visible under the MALDI conditions
used [Several terminated peptides, arising from
impurities in the commercial amino acids used, are
marked with an asterisk (*)]. C. Signature of the
20   _active_ (binding) pool eluted from the C3G-derived
synthetic peptide affinity column. Eight of the
protein analogues displayed appreciable binding under
these conditions, showing that dipeptide sequences
$N^{146}D^{147}$; $D^{147}E^{148}$; $E^{148}E^{149}$; $L^{151}P^{152}$; $I^{161}R^{162}$; $R^{148}D^{149}$; $D^{153}K^{154}$; and,
25     $K^{144}P^{145}$ could be replaced by the dipeptide analogue
without significant loss of activity. [Dipeptide
sequences in parentheses, viz.   '(ED)' & '(DL)',
indicates the notable Gly-[COS]-ßAla-containing
protein analogues not showing significant binding
30   activity].

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Figure 26 illustrates a readout of the composition of
the parent nine component cCrk SH3 domain array, and
the binding and non-binding pools.. The cCrk SH3
domain array of protein analogues was folded in assay
5   buffer and added to a C3G peptide affinity column.
Column fractions were treated with hydroxylamine and
then analyzed by MALDI mass spectrometry. A.
(control) Composition of the parent array of cCrk SH3
domain analogues. B. (Wash) Non-binding cCrk SH3
10   domain analogues eluted in the 0.5 M NaCl wash. C.
(Elution) Specifically-bound cCrk SH3 domain
analogues eluted with hydroxylamine. MALDI peaks are
marked by the single letter code for the dipeptide
that had been was substituted with Gly-SßAla.

15   Figure 27 illustrates an iterative protein signature
analysis applied to the N-terminal SH3 domain of
murine c-Crk. (Top) The amino acid sequence of the 58
residue polypeptide chain is shown. Protein signature
analysis was used to study how chemical variation of
20   the centrally-located ten residue region (highlighted
residues 156-165) affected C3G peptide binding. Two
rounds of signature analysis were performed, using
different dipeptide analogue units. A. Round 1.
Signature of the active (binding) pool obtained from
25   the nine-membered array of Gly-[COS]-Gly-containing
protein analogues. In contrast to the previous
experiment, analysis of this signature reveals that
all_nine protein analogues were present in the
binding pool. B. Round 2. Signature of the active
30   (binding) pool resulting from passing the parent
array of Gly-[COS]-ßAla-containing protein analogues

over a C3G-derived synthetic peptide affinity column.
The signature data shown represents an expansion of
the larger signature shown in Figure 25C. Only four
dipeptide sequences out of a total of nine ($I^{140}R^{142}$;
5    $R^{142}D^{143}$; $D^{143}K^{144}$; and, $K^{144}P^{145}$) in this region, could be
replaced by Gly-[COS]-βAla without significant loss
of binding activity.

Figure 28 illustrates a characterization of the
purified synthetic murine cCrk 134-191, N-terminal
10   SH3 domain. A. Analytical HPLC of the total crude
peptide products from HF cleavage. B. Analytical
HPLC of the purified product on a gradient of 20%-
50%B over 40 minutes. C. Electrospray mass spectra
of the purified product. Inset spectrum is
15   reconstructed to a single charge state from the raw
data below. Calculated mass for $C_{313}H_{470}N_{84}O_{95}S_1$ 6961.8 Da
(average isotope distribution); Observed mass 6962±1
Da.

Figure 29 illustrates a characterization of the
20   parent array of synthetic analogues of the cCrk SH3
domain. A) Reverse phase HPLC analysis of the crude
nine component protein array. B) MALDI mass spectrum
of the same crude product mixture. The protein array
contained predominantly full length polypeptide
25   products. The presence of lower molecular weight
species in the MALDI spectrum result from termination
reactions during chemical synthesis. A') Treatment
of the array with hydroxylamine produces the cleaved
peptide products. Reverse phase HPLC of the mixture
30   after chemical cleavage with NH$_2$OH showed partial

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resolution of the 18 peptide fragments generated.
B') MALDI mass spectrometry of the same cleaved
mixture showed the characteristic patterns of
cleavage fragments. The peaks marked

5   (•)unambiguously identified the protein components
present in the original mixture. The order of these
peaks in the mass spectrum identifies the
corresponding analogue in the parent array and
defines the position of the analogue unit in the

10 polypeptide sequence.

Figure 30 illustrates affinity chromatography
performed on the nine component cCrk SH3 domain
protein analogue array as monitored by UV absorbance
at 280 nm. The 0.5 M NaCl wash eluted all non-

15 specific binding protein analogues as shown by
absence of significant elution with 1 M NaCl.
Protein analogues able to bind to the agarose-bound
C3G derived synthetic peptide were then eluted by
cleavage by hydroxylamine of the thioester bond in

20 each polypeptide chain. This procedure resulted in a
"functional selection" among the array of protein
analogues, giving a binding pool and a non-binding
pool. As a control, a column derivatized with a non-
specific peptide, derived from [Leu⁵] Enkephalin was

25 substituted for the C3G column. As shown, in the
hatched peaks, the entire SH3 protein array eluted
with the 0.5 M NaCl wash and no specific binding was
observed.

Figure 31 illustrates a region of the three-

30 dimensional structure of the c-Crk-C3G complex,

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showing the three acidic residues within the RT loop
of the SH3 domain interacting with Lys' of the bound
C3G peptide ligand. [Taken from a crystal structure].
These interactions are believed to make an important
5   contribution to binding, and to play a critical role
in orienting the interaction of c-Crk with C3G.

Figure 32 illustrates results from two rounds of
protein signature analysis of the sequence comprising
residues 156-165 superimposed on the crystal
10   structure of the N-terminal c-Crk SH3 domain
complexed to the proline rich C3G peptide. Indicated
are those regions of the polypeptide chain observed
to be either tolerant (green) or intolerant (red) of
an extra backbone methylene group

15   Figure 33 represents a computer generated model of
the SH3 domain. The green + red regions represent
the perturbed sites of a peptide ladder fragment from
a section of the SH-3 domain. The green molecular
region represents that a perturbation in this region
20   has no effect on the activity with the ligand which
is depicted in yellow. The red molecular region
represents that a perturbation of this region
destroys activity with the ligand. The gray region
represents the remaining unperturbed (unanalyzed)
25   regions of the SH3 domain. The yellow molecular
region represents a proline rich peptide ligand.

Detailed Description of the Invention

The invention is directed to a 3 step
methodology, titled Protein Signature analysis, for

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the identification of active or inactive components
of a protein. The first step involves a
combinatorial method for synthesizing a peptide
ladder library corresponding to a protein, protein
5   fragment, or other bioactive peptide. The second
step comprises a method for screening the peptide
ladder library with respect to a binding function.
The third step comprises a method for identifying
active or inactive components of the peptide.
10   Successive iterations of the method can be employed
to obtain a complete deconstructive analysis of a
protein, even if the structure of the protein is
unknown. The invention may be employed to
characterize protein interactions and can facilitate
15   the design of new therapeutics which are dependent
upon such protein interaction.

The methodology combines the control of peptide
composition provided by multiple synthesis of
individual peptides with the synthetic convenience of
20   a split and recombine synthetic strategy. By
synthesizing an array of peptides which differ from a
parent molecule by a limited number of defined
modifications, the contribution of specific molecular
features to peptide function can be probed in a
25   systematic manner.

The methodology further comprises a novel
encoding scheme which allows for the array of
synthetic peptide analogues to be assayed free in
solution. The composition of the peptide mixture can
30   then be determined by a single readout operation.

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The methodology was used to synthesize an array
of peptide analogues in which a specific modification
was systematically incorporated into unique positions
in a peptide sequence (examples 1,2 and 3 infra).
5   The synthesis was carried out in such a way that the
resulting mixture contained a defined family of
modified peptides, with each peptide molecule
containing only a single modification. The position
of the analogue moiety within each member of the
10   array was self-encoded by incorporating a selectively
cleavable bond into the analogue structure.

The synthetic polypeptide array was folded and
analyzed for ligand binding on an affinity column as
a single mixture, producing two separate binding and
15   non-binding pools of protein analogues.

Following selective cleavage of each
polypeptide chain at the site of modification, the
resulting mixture of peptide fragments (either the
binding or non binding pool) was analyzed by MALDI
20   mass spectrometry to generate patterns of data which
defined the presence or absence of each peptide
analogue in the ligand binding and non-binding pools.
This mass spectrometric signature related the
position of the chemical modification in the
25   polypeptide sequence to the ability to fold and/or
bind to a specific ligand for the protein (examples
1,2 and 3, infra).

Example 1 (illustrates chemical synthesis and readout
of self encoded arrays of peptide analogues -
30   functional separation step is not included here)

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A. <u>Strategy for the Preparation of a Defined Array of Peptide Analogues as illustrated in Figure 4.</u>

A peptide analogue array for use with the proposed self-encoding scheme has two important features. First, the components of the array must be present in approximately equimolar amounts. Second, to avoid ambiguities, the array should consist only of peptides containing a <u>single</u> chemical modification per peptide chain, at a defined number of positions in the sequence. A straightforward procedure for synthesizing an array of this type has been developed and is schematically represented in Figure 4. For simplicity, the procedure will be illustrated for a hypothetical array of peptides consisting of substitutions of a single amino acid analogue at each of ten consecutive positions in the amino acid sequence of the parent peptide.

Two manual solid phase peptide synthesis (SPPS) reaction vessels, A and B, and a small fritted funnel, 1, are used to manipulate the peptide-resin. The synthesis begins with ten units of peptide-resin in vessel A. After deprotection of the α-amino group, one unit of peptide-resin is removed from A and added to 1. The first amino acid is then coupled to the nine units of peptide-resin in A and the analogue moiety to the one unit peptide-resin sample in 1. After the coupling step, the analogue-modified peptide-resin from 1 is transferred to B.

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To initiate the next cycle of synthesis, the
peptide-resin in vessels A and B are deprotected.
Then another unit of peptide-resin is removed from A
and transferred to the now empty 1. The next amino
5   acid in the sequence of the parent peptide is added
in activated form to both A and B, while the analogue
moiety is reacted with the new peptide-resin sample
in 1. After completion of this cycle, the modified
peptide-resin in 1 is added to B. The synthesis
10   continues in this manner for the requisite ten
cycles.

Throughout the synthesis, vessel A contains only
unmodified peptide-resin. Vessel B contains all
single-site modified peptide-resins and vessel 1
15   contains the current sample of peptide-resin which is
being modified. All chemical steps carried out in
vessels A and B are identical, adding the amino acids
of the unmodified sequence. At the end of 10 cycles,
all the resin in vessel A has been transferred into
20   vessel B which now contains the desired array of
peptide analogues in resin-bound form.

B.   Synthesis of a Defined Peptide Array.

A peptide array consisting of a ten amino acid
sequence, GDILRIRDKP was chosen as a target to
25   demonstrate the approach, following the methodology
as described above. The target array is shown in
Figure 15A, and consists of overlapping dipeptide
analogues in the region of interest. In order to
facilitate characterization by mass spectrometry, the

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array was synthesized on resin bearing the sequence
EEAcpRLKLKAR, where Acp is ε-aminocaproic acid (Zhao
et al. *Proc. Nat. Acad. Sci.* 1996, *93*, 4020-4024).
The dipeptide analogue moiety corresponding to -NH-
5   CH_2-CO-S-CH_2-CH_2-CO- (Gly-SβAla) was introduced as Boc-
Gly-SβAla. Since the analogue moiety was
incorporated as a <u>dipeptide</u>, a modification was made
to the synthetic procedure outlined above and shown
in Figure 4. In order to keep the synthetic
10   operations being performed on the peptides in vessels
A and B in register, the sample being derivatized in
1 was held out for <u>two</u> cycles before transfer to
vessel B. To accommodate this modification, a second
auxiliary funnel 1' was added.

15   In practice, the peptide-resin sample from
vessel A was added to a funnel in position 1, where
the dipeptide analogue coupling was initiated. After
one cycle, the funnel was moved to position 1', where
the dipeptide analogue coupling continued during a
20   second cycle of chain elongation in vessels A and B.
The analogue-containing sample of peptide-resin was
then washed with DMF (dimethylformamide) and
transferred to vessel B. The synthetic steps for
this synthesis are outlined in Figure 15B. After
25   substituting dipeptide analogues for nine consecutive
dipeptide sequences spanning a region of 10 amino
acids, four additional amino acids, PFKK, were
coupled to the array of peptide-resins in vessel B to
complete the target sequence.

30   C. <u>Characterization of the Peptide Array.</u>

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The peptide array described above contains a mixture of nine peptides, all 24 residues in length, each differing only in the position of a Gly-SßAla dipeptide substitution. As expected, the analytical HPLC of this array is quite complex, with many overlapping peaks (Figure 16A) The MALDI mass spectrum is also poorly resolved since the peptides in the array have a high redundancy in their molecular weights (Figure 16B). Thus the sequence -LRIRD- contains the dipeptides LR, RI, IR, each of which have a molecular weight of 269 Da and RD which has a molecular weight of 271 Da. When substituted with Gly-SßAla (145 Da) in the peptide arrays, each of these substitutions would result in a peptide analogue with a molecular weight of 125±1 Da below that of the unmodified sequence. (PFKK-GDILRIRDKP-EEAcpRLKLKAR·amide, M.W. 2920 Da). The resulting MALDI mass spectrum of this peptide array would be expected to have a large peak around 2795 Da, representing the sum of four different peptide components, (see Figure 16B).

D.   'Self-Encoded' Peptide Arrays.

The poor HPLC separation and redundancy in molecular weight creates a challenge for identification of components present in the array of peptide analogues. The distinguishing feature of the components in this peptide array is the unique position of the modification in the sequence. One approach to the unambiguous identification of the peptide components is to incorporate a selectively

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cleavable bond in the analogue unit. Cleavage of
this bond in an analogue peptide would result in two
peptide fragments whose lengths, measured as mass,
would define the position of the analogue unit in the
5   peptide from which they derived. Such a chemical
cleavage site would have to be stable to normal
handling (folding and assay) conditions, while
permitting selective cleavage on demand. We have
investigated the incorporation, stability and
10   selective cleavage properties of two potential
readout chemistries, based on ester and thioester
bonds.

         1.   Synthesis and Characterization of
               a Peptide Containing a Cleavable
15   Thioester Backbone as illustrated
               in Figure 17A.

         The peptide LYRA(Gly-SβAla)-YGGFL·amide, was
synthesized by stepwise SPPS using *in situ*
neutralization coupling protocols. The thioester-
20   containing dipeptide analogue, Boc-Gly-SβAla was
activated as an HOBt ester and then coupled to pre-
neutralized NH₂-YGGFL-(4-Me)benzhydrylamine)-resin.
Following deprotection and cleavage from the peptide-
resin, the stability of the model thioester-
25   containing peptide was determined.

          Thioester bonds within peptide sequences have
been found to be stable at neutral pH (Schnölzer et
al. *Science* **1992**, *256*, 221-225; Baca et al. *J. Am.
Chem. Soc.* **1995**, *117*, 1881-1887; Canne et al. *J. Am.*

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Chem. Soc. 1996, *118*, 5891-5896). To test for
stability to base hydrolysis, the peptide was
dissolved at pH 9.0 in 200 µL of 100 mM Tris, 1 M
Gn·HCl, vortexed vigorously for 10 seconds and left at
5   23°C for 30 minutes. Surprisingly, no hydrolysis was
observed under these conditions. Addition of 20 µL
of 1 M NaOH to (pH~13) gave complete hydrolysis after
just 10 minutes as monitored by HPLC and electrospray
mass spectrometry. In contrast to their stability to
10   hydrolysis, thioesters have been shown to be very
labile to hydroxylamine at neutral pH levels (Bruice
et al. *J. Am. Chem. Soc.* 1964, *86*, 4886-4897). As
shown in Figure 17A, the thioester peptide was
completely cleaved into LYRAG-NH_2OH and $HSCH_2CH_2CO$-
15   YGGFL·amide when dissolved in 1 M NH_2OH, 200 mM NH_4HCO_3,
pH 6.0 for 30 minutes. Thioesters can be completely
cleaved at concentrations of NH_2OH as low as 10 mM in
< 30 minutes.

        2.   <u>Synthesis and Characterization of a</u>
20   <u>Peptide Containing a Cleavable Ester</u>
              <u>Backbone as illustrated in Figure 17B</u>.

        The peptide, YKLFAla-[coo]-YGGFL·amide was
prepared by stepwise SPPS, using *in situ*
25   neutralization coupling protocols and Boc chemistry.
The ester bond in the peptide was formed by coupling
Boc-Ala to an α-hydroxy acid using 4-
dimethylaminopyridine as a catalyst. Following
deprotection and cleavage from the peptide-resin, the
30   model ester-containing peptide was analyzed for

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stability. The ester bond was quite resistant to
hydrolysis, taking six hours to cleave at pH 10,
Figure 17B. In addition, the ester peptide was
stable to treatment with 1 M NH_2OH, 200 mM NH_4HCO_3, pH

5   6.0 for up to 12 hours. The high stability of the
ester to hydroxylamine should allow for the use of a
backbone thioester as a chemical readout in peptides
containing ester bonds. More recent studies have
shown that the ester can be readily cleaved by NH_2NH_2

10   at neutral pH. A Gly-[coo]-Gly containing peptide was
cleaved in under one hour by dissolving in 150 mM
hydrazine, 100 mM Sodium Phosphate, 6 M guanidine·HCl,
pH 7.0 (Carrasco, M. unpublished).

        E.   Readout of the nine component peptide
15   analogue array of the parent sequence PFKK-
             GDILRIRDKP-EEAcpRLKLKAR·amide.

        Synthesis of this array has been described
above. Each member of this peptide array contains a
Gly-SßAla replacement at one of the nine possible
20   dipeptide positions within the ten amino acid
sequence, [GDILRIRDKP] as shown in Figure 15A. In
order to facilitate unambiguous identification of the
components in the array, the thioester readout
chemistry has been incorporated into the dipeptide
25   analogue. The thioester bond in the Gly-SßAla
dipeptide analogue introduces a unique cleavage site
into each member of the peptide array. Chemical
cleavage of the peptide analogue array was expected
to produce 18 peptide fragments as shown in Figure
30   18.

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The peptide analogue array was cleaved by
treatment with 1 M NH$_2$OH, 200 mM NH$_4$HCO$_3$, pH 6.0 for
20 minutes. The resulting peptide fragments were
then analyzed by HPLC and by MALDI mass spectrometry.
As with the uncleaved peptide array, Figure 16A, the
components of the cleaved peptide array, Figure 16C,
still give rise to a complicated and essentially
uninformative HPLC chromatogram. By contrast, the
MALDI spectrum of the unfractionated cleaved peptide
array, provides a very straightforward
characterization of the peptide array, Figure 16D.
As shown in Figure 19, the masses of the nine C-
terminal fragments from the cleaved array are easily
located in the mass spectrum (Signals corresponding
to 5 of the 9 N-terminal peptides were also
observed. The masses that were not resolved were
obscured by matrix ions in the region below 1000 Da
in the spectrum of the peptide mixture), and served
to unambiguously identify the position of the
analogue unit in each component of the original
peptide array before cleavage.

The MALDI mass spectrometric readout
characterized the peptide array in several ways.
Cleavage of the analogue unit, at different positions
throughout the array, produces two families of
related peptides. These two families have either the
N- or C-terminus of the parent peptide in common. In
this experiment, the identification of each member of
the C-terminal family of peptides by MALDI can be
directly related to the presence of each full length
peptide analogue in the parent array. In addition to

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the C-terminal family, five members of the N-terminal
family of peptides were also observed. The starred
peaks on mass spectrum in Figure 16D correspond to
these N-terminal peptides. In practice, the
5   identification of either the N-terminal or C-terminal
peptide fragments would serve to unambiguously
characterize the peptide analogue array.

Another characterization of the peptide array
can be obtained by looking at the difference in
10   masses between the peaks on the mass spectra. As
shown in Figure 19, the nine C-terminal peptide
fragments are all of different lengths, and the mass
differences between neighboring fragments correspond
to the masses of individual amino acid residues. By
15   correlation of these mass differences to the mass of
individual amino acids, the sequence through which
the analogue unit was substituted can be confirmed.
This type of analysis has been previously used to
sequence native peptides by 'protein ladder
20   sequencing' (Chait et al. *Science*. **1993**, *262*, 89-92).

F.   Discussion

An embodiment of the present invention is the
synthesis of a defined array of peptide analogues as
a single mixture. This peptide array can be assayed
25   as a pool, after which individual peptide components
can be identified through a novel encoding scheme.
Using this approach, the comparative properties of
the individual components can be determined for a
given assay/function. The adaptation of this

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analogue array methodology to the study of structure-
function relationships in proteins is illustrated in
Example 2 (infra).

The peptide array can be synthesized using a
modified 'split resin' procedure which, unlike
previous procedures, results in a defined array of
components with only the desired complexity. A key
aspect of this approach is the ability to prepare a
defined subset of a fully combinatorial synthesis.
By synthesizing such a subset, arrays of manageable
size can be prepared that contain defined
modifications at a large number of positions in the
peptide sequence. For example, using this
methodology, five different amino acid analogues
could be substituted at each position of a ten amino
acid sequence so that there is only one modification
in each peptide molecule. The resulting array would
result in a mixture of 50 peptides (5 analogue
structures x 10 positions). Using the standard split
synthesis approach (Furka et al. *Int. J. Pept. Prot.
Res.* **1991**, 37, 487-494; Lam et al. *Nature* **1991**, *354*,
82-84), the same 50 peptides would be only a small
fraction of a library of ~10' (5^{10}) peptides.

Once diversity has been generated, and a
selection performed, a method is needed to identify
individual components. Such approaches to decoding
peptide mixtures have presented a substantial
challenge. Most encoding strategies involve a
molecular tag which can be read by sensitive
analytical techniques (Needels et al. *Proc. Natl.*

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Acad. Sci. U.S.A. **1993**, *90*,10700-10704; Kerr et al.
J. Am. Chem. Soc. **1993**, *115*, 2529-2531; Nikolaiv et
al. *Pept. Res.* **1993**, *6*, 161-170; Ohlmeyer et al.
Proc. Natl. Acad. Sci. U.S.A. **1993**, *90*, 10922-10926)

5   or by amplification (Brenner et al. *Proc. Natl. Acad.
Sci. USA. **1992**, *89*, 5381-5383; Nielsen et al. *J. Am.
Chem. Soc.***1993**, *115*, 9812-9813). Many of these
techniques, however, rely on the assay of molecules
still attached to a solid support and the isolation

10   and analysis of individual beads. To avoid the
necessity for a solid support, the encoding must be
associated with the peptide analogue at the molecular
level (Brenner et al. *Proc. Natl. Acad. Sci. USA.*
1992, *89*, 5381-5383; Nielsen et al. *J. Am. Chem.

15   Soc.***1993**, *115*, 9812-9813).

       Incorporation of a chemically cleavable bond at
specific sites within the peptide analogues provides
an example of an alternative simple and practical
encoding scheme at the molecular level. After a

20   physical selection for a desired function, the
components of the peptide array can be decoded
through chemical cleavage and one step mass
spectrometric readout. Detection of the resulting
peptide fragments unambiguously defines the presence

25   or absence of a given analogue molecule in the
selected population. As demonstrated in the analysis
of the nine member peptide array, matrix assisted
laser-desorption ionization (MALDI) mass spectrometry
is well suited for the decoding of linear arrays of

30   peptides (Chait et al. *Science.* **1993**, *262*, 89-92).

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This use of mass spectrometry to decode mixtures of peptide analogues is analogous to the use of gel electrophoresis to separate nucleotides by length during DNA sequencing and analysis (A similar cleavage and separation by size readout was used for nucleic acids in: Pan et al.; *Science*. 1991, *254*,1361-1364; Hayashibara et al. *J. Am. Chem. Soc.* 1991, *113*, 5104-5106). The high resolution and sensitivity (<1 pmol/component; Chait et al. *Science* 1992, *257*, 1885-1894) of MALDI mass spectrometry allows the characterization of even small quantities of the entire peptide array.

In this example, we have demonstrated the feasibility of this approach through the synthesis of a peptide array which was then characterized by MALDI mass spectrometry following chemical cleavage. A nine component peptide array of the parent sequence, PFKK-GDILRIRDKP-EEAcpRLKLKAR·amide, was synthesized in which a single dipeptide analogue, Gly-SßAla, was introduced into consecutive positions through the sequence -GDILRIRDKP- (Figure 15). This array was self-encoded with a chemically cleavable thioester bond which was incorporated into the analogue unit. The peptide components were then identified by cleaving the thioester bond in each peptide with hydroxylamine, followed by MALDI mass spectrometry. The resulting series of peaks on the mass spectrum unambiguously identified the presence of all nine peptide analogues in the peptide array.

The combination of the synthesis of an array of

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peptides corresponding to a defined subset of a fully
combinatorial mixture with a self-encoding strategy
results in an information-rich approach to the
elucidation of the structure-activity relationship of
5   peptides, polypeptides and proteins. The power of
this approach is illustrated when the peptide array
is subjected to a functional selection (example 2).
Since all members of the peptide array can be
observed in a single readout step, this approach can
10 generate information on both the positively and
negatively selected members of the array. It is
anticipated that the ability to synthesize multiple
peptide analogues in a single procedure, followed by
functional characterization of the entire peptide
15 array will give greater insight into the molecular
basis of peptide function.

This procedure allows for controlled diversity
to be generated at multiple positions in a peptide
chain. In addition, a novel self-encoded approach to
20 identifying peptide analogues has been developed
which involves the incorporation of a cleavable bond
which is associated with a particular modification.
This chemical readout system allows the entire
peptide array to be analyzed simultaneously by
25 sensitive analytical techniques such as MALDI mass
spectrometry.

By reading out an entire pool of peptide
analogues in a single step, a profile of structure-
function relationship across all members of the array
30 could be generated. Interpretation of such profiles

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will provide information of the molecular basis for
peptide function. Such peptide arrays could be used
to elucidate the molecular basis of important
functional properties by systematically removing
5   structural elements of a peptide. In particular,
hydrogen bond donors and acceptors in the backbone
can be deleted using a wide variety of backbone
analogues. Additionally, new functional
characteristics can be introduced through the
10   systematic introduction of new sidechain and backbone
groups. The use of defined arrays of synthetic
peptide analogues coupled with a single step readout
provides new insights into the chemical basis of
peptide activity.

15

Example 2 (illustrates chemical synthesis, functional
separation, and readout of self-encoded arrays of an
SH3 peptide consisting of the ten amino acid
sequence, GDILRIRDKP)

20   This example illustrates an integrated approach to
the preparation of a defined array of protein
analogues in a single synthesis, their functional
separation into active and inactive pools, and a
simple one step readout of the composition of the
25   self-encoded mixtures. The strategy is outlined in
Figure 21. The chemical synthesis and encoding
strategies are an extension to proteins of the
approach described in example 1 (supra). Instead of
synthesizing a single modified protein, an array of
30   protein analogues is prepared in a single total

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chemical synthesis. The proteins in the array differ
from each other only by the position in which a
defined covalent modification is located within the
polypeptide sequence. The composition of the
analogue array can be decoded by means of a latent
readout chemistry introduced in conjunction with each
analogue unit. This latent readout chemistry allows
proteins to be specifically cleaved, yielding a
pattern of peptide fragments which unambiguously
identifies the individual components of the mixture
and defines the position of the analogue unit in each
compound (A similar readout system has been developed
for use in DNA systems: Hayashibara et al. *J. Am.
Chem. Soc.* 1991, *113*, 5103-5106). By comparing the
readout patterns obtained before and after a
functional separation, a profile is obtained relating
the effects of the analogue structure to its position
in the polypeptide chain. Accumulation and
interpretation of such qualitative profiles of
protein structure-function relationships 'protein
signatures' provides insights into the chemical basis
of protein function.

A family of analogue proteins was characterized
using the invention with the N-terminal SH3 domain of
the adapter protein cCrk (Murine cCrk, N-terminal SH3
domain (residues 134-191); Genbank accession s72408).
SH3 domains are small monomeric modules which are
present in many proteins involved in signal
transduction. It is now well established that SH3
domains mediate protein-protein interactions within
intracellular signaling networks through the

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recognition of short proline-rich sequences from
other adapter proteins (Ren et al. *Science* 1993, *259*,
1157-1161).   Individual SH3 domains have been shown
to fold *in vitro* to a defined tertiary structure and
5   to bind proline-rich peptides with low μM affinity.
In addition several of these domains have been
structurally characterized by both NMR and X-ray
crystallography (Musacchio et al. *Nature* 1992, *359*,
851-855; Yu et al. *Science* 1992, *258*, 1665-1668).

10   Using the methods described in example 1
(supra), a family of nine analogues was prepared to
investigate the effect of an extensive covalent
modification of the 58 amino acid residue SH3
polypeptide chain on its ability to fold and bind its
15   specific peptide ligand.   Each member of the
synthetic protein array contained a single dipeptide
analogue unit, $-NH-CH_2CO-SCH_2CH_2CO-$ (Gly-SßAla),
replacing pairs of adjacent amino acids at unique
positions in the native sequence (see Figure 23).
20   The mixture of analogue polypeptide chains was folded
and assayed for binding to a specific ligand, a short
proline-rich synthetic peptide derived from the
sequence of the guanine nucleotide exchange factor
C3G (Knudsen et al. *J. Biol. Chem.* 1994, *269*, 32781-
25   32787).   After the affinity selection, the binding
and non-binding pools of protein analogues were
cleaved selectively at the thioester bond contained
in each analogue unit, and the composition of each
pool was read out using MALDI mass spectrometry.   In
30   this manner, a pattern of signals was obtained, which
related the position of the chemical modification

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within the SH3 polypeptide sequence to its effects on folding and/or ligand binding.

A. Chemical Synthesis of the 58 residue SH3 polypeptide

5   The polypeptide chain of the murine cCrk N-terminal SH3 domain, corresponding to residues 134-191 of the full cCrk signaling protein (Murine cCrk, N-terminal SH3 domain (residues 134-191); Genbank accession s72408), was assembled by highly optimized,
10   stepwise solid phase peptide synthesis using machine-assisted *in situ* neutralization protocols for *tert*-butoxycarbonyl (Boc) chemistry (Schnölzer et al. *Int. J. Pept. Protein Res.* 1992, *40*, 180-193). After deprotection and cleavage from the resin support, the
15   crude polypeptide was purified by semipreparative reversed-phase HPLC and lyophilized using procedures as described by Clark-Lewis et al. *The Use of HPLC in Receptor Biochemistry*; Venter, J.C. and Harrison, L.C. Eds.; Alan R. Liss, Inc: New York, 1989; Chapter
20   3. The purified product was characterized by analytical HPLC and by electrospray mass spectrometry (Schnölzer et al. *Anal Biochem.* 1992. *204.* 335-343). The results are shown in Figure 28.

B. Functional characterization of the
25   synthetic SH3 domain

The N-terminal cCrk SH3 domain was formed by folding under these conditions: 0.2 mg of the purified 58 residue polypeptide in 600 μL of 20 mM

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HEPES 50 mM NaCl pH 7.3 at room temperature for 15 minutes. The folded protein was structurally characterized by NMR and crystallization (The folded protein was characterized by two dimensional NOESY 1H NMR spectroscopy. In addition, the synthetic protein solution used for NMR analysis spontaneously formed crystals upon storage at 4°C. These crystals diffracted to >2.3 Å allowing for the solution by molecular replacement. Refinement of the structure is in progress). The resulting synthetic protein domain was then assayed for its affinity for two different proline-rich peptides. Binding of this SH3 domain to its cognate peptide ligand buries a tryptophan sidechain of the protein found in the binding pocket. This change in solvent exposure leads to an increase in the fluorescence intensity of the tryptophan sidechain which can be monitored as a function of increasing ligand concentration (Feng et al. *Science* 1994.*266*, 1241-1245). The K_d's for the peptides C3G (PPALPPKKR·amide) and a peptide designed for attachment to an affinity column, Acetyl-CWAcp-C3G, were found to be 1.8 μM and 2.4 μM respectively (Due to the polyproline nature of the ligands, fluorescence measurements were taken after 12 hours incubation of the protein with the peptide ligand to allow equilibration of the multiple cis and trans isomers). The C3G affinity of 1.8 μM for the C3G derived peptide is comparable to the affinity reported for a recombinantly derived SH3 domain (1.9 μM) (Wu et al. *Structure* 1995, *3*, 215-226).

C.   Affinity chromatography of the synthetic

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SH3 domain

An affinity column carrying a synthetic C3G
derived peptide was prepared by reaction of the
cysteine side chain of Acetyl-CWAcp-PPALPPKKR·amide

5   with an iodoacetylated agarose matrix. The linker
Acetyl-CWAcp- was designed to combine a reactive
sulfhydryl on the Cys sidechain with a spectroscopic
tag in the Trp residue, and to introduce a flexible
spacer region between the support and the peptide

10   ligand in the form of ε-aminocaproic acid. The
amount of peptide on the column, was determined as 5
μmol/mL of the swollen agarose matrix from the
absorbance at 280 nm of the peptide solution before
and after reaction with the column. As a control for

15   non-specific binding effects, a second column with
comparable peptide loading was made by the same
procedure using a non C3G sequence, Ac-
CWAcpYGGFL·amide.

Specific binding of the native sequence

20   synthetic cCrk N-terminal SH3 domain was demonstrated
to the C3G-peptide affinity support by loading crude
synthetic cCrk(134-191) mixed with BSA. The proteins
were allowed to equilibrate for 6 hours after which a
series of increasing washes up to 1 M NaCl, and one

25   wash with 1 M NH₄OH was performed. The column
effluent was monitored by absorbance at 280 nm and
selected fractions subjected to mass spectrometry.
The BSA was completely washed off the column after
the 300 mM NaCl wash. The cCrk N-terminal SH3

30   domain, however, remained bound to the column

throughout the NaCl and NH$_2$OH washes. The cCrk domain
was only eluted by a 6M Gn·HCl wash that disrupted the
specific interactions between the protein and the
synthetic C3G-derived peptide. It is interesting to

5   note that the 58 amino acid cCrk SH3 domain was
stable to 1 M NH$_2$OH treatment despite the presence of
the sequence Asn-Gly-Asn (144-146) which can be prone
to NH$_2$OH cleavage (Clarke et al. *Stability of Protein
Pharmaceuticals, Part A:*, Ahern, T. J.; Manning, M.C.

10   Ed. Plenum Press, New York, 1992).

D.   Chemical synthesis of an array of analogues
of the 58 residue polypeptide chain

The target array consisted of nine polypeptides,
each containing a single -NHCH$_2$CO-SCH$_2$CH$_2$CO- (Gly-

15   SßAla) substitution at one of the nine possible
dipeptide units within the ten amino acid sequence
defined by residues 156-165 within the cCrk sequence
134-191 (Figure 23). The Gly-S-ßAla analogue unit
was designed to remove two consecutive amino acid

20   sidechains and to insert an extra methylene into the
polypeptide backbone of the SH3 protein. Inclusion
of the thioester bond added a latent chemical
cleavage site for the readout of the composition of
the mixture of protein analogues and simultaneously

25   deleted a backbone hydrogen bond donor. Backbone
flexibility was also increased due to the loss of the
planarity associated with the peptide bond. The
analogue substitutions covered overlapping dipeptide
sequences through the region -GDRILRIDKP-,

30   corresponding to residues 156-165 of the cCrk

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sequence as shown in Figure 23A.

    The polypeptide analogue array was synthesized
using a combination of manual and machine-assisted
protocols. The sequences corresponding to cCrk(166-
5   191) and cCrk(134-155) were synthesized using a
machine-assisted protocol for Boc-solid phase
chemistry (Schnölzer et al. *Int. J. Pept. Protein
Res.* 1992, *40*, 180-193). Following synthesis of
cCrk(166-191), the peptide-resins were removed from
10   the peptide synthesizer and placed in a manual-
synthesis reaction vessel. Synthesis of cCrk(156-
165) with concomitant introduction of the analogue
unit at each position was performed as previously
described for a model peptide system (A similar
15   readout system has been developed for use in DNA
systems: Hayashibara et al. *J. Am. Chem. Soc.* 1991,
113, 5103-5106) using a modified split-resin
procedure. Before addition of each residue (157-
165), a sample of peptide-resin was removed from
20   reaction vessel A, modified with a dipeptide analogue
for two synthetic cycles and then transferred to a
second reaction vessel B. Identical synthetic
operations building up the native amino acid sequence
were carried out in reaction vessels A and B. In
25   this manner, an array of nine resin-bound
polypeptides was created as a single mixture,
containing consecutive overlapping Gly-SßAla
substitutions. Chain elongation of the nine
component mixture was continued through the sequence
30   cCrk(134-155) using machine assisted synthetic
cycles. The mixture of full-length analogue-

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containing polypeptide-resins was subjected to HF
cleavage and simultaneous sidechain deprotection to
give a crude lyophilized mixture of nine analogues of
the 58 amino acid polypeptide chain of the N-terminal
5   cCrk SH3 domain.

The members of the polypeptide array were folded
by dissolving 0.5 mg of crude peptide product in 200
μL of 20 mM HEPES 50 mM NaCl pH 7.3 at room
temperature. After 1 hour, the protein mixture was
10   analyzed by HPLC and MALDI mass spectrometry. By
HPLC (Figure 29A), the nine protein components were
partially resolved against a background of synthetic
byproducts. MALDI mass spectrometry (Figure 29B)
showed an unresolved mixture of full-length
15   polypeptide chains centered around 6950 Da; minor
amounts of terminated components formed as byproducts
in the chain assembly were also present. The
presence of full length protein analogues indicates
that the thioester-containing polypeptide chains are
20   stable under these assay conditions. However,
neither HPLC nor MALDI-MS was able to define the
composition of the array of protein analogues.

E.   Readout of the Composition of the Parent
Array of Protein Analogues

25   In order to characterize the protein array, the
polypeptide chains were specifically cleaved with
hydroxylamine through nucleophilic attack at the
thioester bond. The resulting peptide fragments were
analyzed by both HPLC and MALDI-TOF, Figures 29A' and
30   29B'. The treatment with hydroxylamine specifically

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cleaved each analogue-containing polypeptide chain at
the site of modification, resulting in a mixture of
peptide fragments. As shown in Figure 23, the
cleavage of the protein array produces a mixture of
5   peptide fragments, each with a different number of
amino acids in the peptide chain. As shown in Figure
29A', reverse-phase HPLC analysis of the cleaved
mixture yields complicated sets of partly unresolved
peaks. In addition, the order of the peaks bears no
10   direct relationship to the position of the analogue
unit in the parent polypeptide chain. Analysis of
the cleaved mixture by MALDI mass spectrometry, on
the other hand, produces a series of well resolved
peaks, the relative positions and masses of which are
15   directly related to the position in which the
analogue unit was placed in each full-length
polypeptide chain. The peptide ladder shown in
Figure 29B' corresponded to all nine of the expected
N-terminal peptide fragments resulting from cleavage
20   of the nine protein analogues, and unambiguously
characterized the protein array.

Since the cleavable bond was placed within the
58 residue SH3 polypeptide chain, the cleavage of
each analogue must produce both N- and C-terminal
25   peptide fragments. In the MALDI spectrum, however,
only the N-terminal peptide fragments were observed
with high intensity. The analysis of peptide
mixtures by MALDI mass spectrometry can vary
depending on the choice of matrix and solvent
30   composition. This phenomena did not compromise our
experimental results since the MALDI readout system

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relied on the comparison of mass spectra before and
after selection. In addition, detection of only one
of the two peptide fragments from each protein was
required for identification of the parent protein
5   analogue. In fact, interpretation can be simplified
when only fragments corresponding to one end of the
polypeptide chain are observed.

       F.   Affinity chromatography of the array of
          cCrk SH3 protein analogues

10   The lyophilized crude mixture of 58 residue
polypeptide chain analogues was folded by dissolving
1.5 mg in 600 μL of 20 mM HEPES, 50 mM NaCl, pH 7.3.
The dissolved protein array was then applied to the
affinity column and left to bind for 6 hours. The
15   column was then washed with 0.5 M NaCl buffer to
remove nonspecific binding proteins from the column.
Specifically bound protein analogues were then eluted
with 1 M hydroxylamine bufffer. Amounts of eluted
peptide were monitored by UV absorbance at 280 nm.
20   This procedure was used for both the C3G peptide
column and for the control column loaded with Ac-C-
AcpYGGFL·amide. The hydroxylamine cleavage of the
thioester bond results in the elution of peptide
fragments corresponding to the proteins which were
25   able to bind to the affinity column under these
conditions. The results obtained are shown in Figure
30. Specific binding was found only for the C3G
peptide column.

       G.   Readout of the composition of the arrays of
30   protein analogues

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The composition of the parent array of SH3 protein analogues, as well as the binding and non-binding fractions obtained from affinity chromatography was determined by chemical cleavage and MALDI MS. First, the parent array of folded protein analogues and the 0.5 M NaCl wash from the affinity chromatography of the array were cleaved separately with hydroxylamine. After a desalting step, MALDI mass spectra were obtained for the peptide fragments generated from the (parent) protein array, the salt wash (non-binding) and for the peptide fragments generated by the hydroxylamine elution of the specifically bound analogues (binding). The results are shown in Figure 26. Nine components were present in the array of protein analogues that was added to the C3G column as one mixture (Figure 26A). Of these nine components, five did not bind significantly to the affinity column and were present only in the 0.5 M NaCl wash fractions (Figure 26B). Three protein analogues, however, were able to bind to the C3G peptide on the column and were eluted only after cleavage with hydroxylamine (Figure 26C). One of the protein analogues can be identified in both the wash and elution spectra, indicating intermediate folding and/or binding properties for this analogue.

The affinity assay used in this experiment did not allow binding effects to be distinguished from folding effects since lack of binding to the affinity column could arise either from failure to fold or from correctly folded material failing to bind. The

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observed pattern of functional and non-functional
analogues covering the sequence 156-165 of cCrk
indicated that in four of the nine positions even
extensive modifications to the chemical structure of
5   the polypeptide chain were not sufficient to prevent
folding and specific ligand binding.

H.   Discussion

The present invention is the first application
of combinatorial synthetic chemistry techniques to a
10   protein target. Straightforward synthetic access to
protein arrays containing a dipeptide analogue Gly-
SßAla has been demonstrated in the context of
residues 156-165 of the cCrk N-terminal SH3 domain
(residues 134-191) of cCrk. A latent readout
15   functionality has been shown to be stable to
conditions of protein folding and ligand binding, yet
is cleavable by brief treatment with 1 M
hydroxylamine. A selection for binding activity
based on the use of a C3G-derived synthetic peptide
20   for affinity chromatography has been developed and
used to analyze the functional properties of the
array of protein analogues. Finally, chemical
cleavage of synthetically introduced latent cleavage
sites followed by MALDI-TOF mass spectroscopy has
25   been used to read out the composition of 'self-
encoded' pools of protein analogues.

The analogue unit used in this study caused the
simultaneous modification of several aspects of the
covalent structure of the polypeptide chain. The
30   Gly-SßAla dipeptide consisted of four alterations

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from the native dipeptide; two sidechain deletions,
an extra methylene in the backbone and a thioester
substituted for the amide bond. By redesigning the
analogue unit in an iterative manner, further insight
5   into the individual components of the modification
can be elucidated. Although the thioester is needed
for the self-encoding strategy used here, the other
modifications can be deleted in the design of a new
analogue unit. For example, using the dipeptide -
10     $NHCH_2CO-SCH_2CO-$ (Gly-SGly) as the analogue unit would
investigate the role of the extra backbone methylene
group. Alternatively, use of Aaa_n-SßAla as an
analogue unit would reintroduce one sidechain of the
two deleted. By designing new analogue units which
15   differ from Gly-SßAla by a single modification, the
contributions of individual modifications to the
polypeptide structure may be investigated after
repetition of the functional selection and decoding
of the new functional and nonfunctional products.

20   To identify the individual components of the
mixture of protein analogues after functional
selection, a strategy has been developed in which the
modified polypeptides are self-encoded. A cleavable
bond was introduced into the polypeptide backbone at
25   the site of modification. In the case of the
analogue unit Gly-SßAla, the cleavable bond was a
thioester which is stable to the conditions of normal
handling, yet can be selectively cleaved by treatment
with NH_2OH at neutral pH. Chemical cleavage of the
30   thioester bond and subsequent analysis of the
resulting peptide fragments by MALDI mass

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spectrometry, gave a series of peaks which
unambiguously defined the protein components present
in the array before cleavage. This encoding system
is especially powerful since all the information is

5   read out in a single step from a pool of molecules
free in solution.

The qualitative nature of the MALDI mass
spectrometric readout of signature analysis
experiments may allow approximate measurements of

10   binding affinities of individual protein analogues.
One solution would be to vary the conditions of the
functional selection to produce a series mass
spectrometric signatures; for example, selecting the
protein array against a series of affinity columns

15   with increasing ligand concentrations. By monitoring
the presence or absence of an individual mass
spectrometric signal over a range of concentrations,
an approximate Kd for the binding could be
determined. Similar analyses could be performed by

20   varying other parameters such as temperature and
Gn·HCl concentration or through an affinity elution
procedure.

The power of a chemical synthesis approach to
the study of proteins is the straightforward access

25   to a wide range of variations in molecular structure.
In this invention, backbone interactions were studied
by deletion of hydrogen bonding and by insertion of
an extra methylene group. However, many other types
of chemical modifications can be introduced using the

30   methodology presented (supra). For example, further

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studies could investigate the ability of the protein
to tolerate restrictions in backbone conformation;
aminoisobutyric acid (α,α dimethyl glycine) residues
are known to restrict Ramachandran space to alpha
5   helical conformations (Marshall et al. *Circ. Res.,
Suppl. II 30 and 31, 1972. 143-150) while insertion
of beta turn mimics into the polypeptide chain can
provide a test for such secondary structural
features. The modifications possible using the tools
10   of molecular biology are good for monitoring
sidechain interactions but, with the exception of
proline, do little to probe the conformational
properties of the peptide backbone. The use of
chemical synthesis allows for experiments which probe
15   the tolerance of *cis* rather than *trans* peptide bond
replacements and the ability of the peptide backbone
to explore "D" Ramachandran space. Such experiments
may give insight into the molecular characteristics
of the peptide backbone. In this manner, the full
20   range of modifications that have been used to
elucidate the structure-function relationships of
peptides can now be applied to proteins.

The signature analysis technique described in
this example is generally applicable to proteins
25   accessible by chemical synthesis (Muir et al. *Curr.
Opin. Biotech.* 1993, *4*, 420-427). With the
introduction of modular chemical ligation techniques,
individual domains, as well as series of these
domains can be investigated in the context of larger
30   protein molecules. Since these domains are the basic
units of protein function, the systematic generation

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of arrays of analogues will allow the tools of
organic chemistry to be used for the elucidation of
the molecular basis of protein function.

Example 3 (illustrates a 20 peptide example of
chemical synthesis, functional separation, and
readout (protein signature analysis) of self-encoded
arrays of an SH3 domain with the sequence)

In this example, the combinatorial protein
signature analysis has been applied to the N-terminal
SH3 domain from c-Crk. A total of 28 chemically
defined protein analogues were analysed in only two
protein signature analysis experiments. Using protein
signature analysis, the effect on biological function
of modifiying both amino acid side-chains and the
polypeptide backbone of the protein was determined.
The latter of these, i.e. systematic backbone
engineering, is unprecedented in the study of
proteins. Protein signature analysis provides a
framework for the systematic application of chemistry
to deciphering how proteins work, and thus
complements the analogous chemical approaches already
available for the study of nucleic acids (Min et
al.(1996) *J. Am. Chem. Soc.*118, 6116-6120).
Consequently, fundamental biological processes such
as protein folding, binding, and catalysis come under
the scrutiny of synthetic organic chemistry.

In the form shown in Figure 20, protein
signature analysis is a particularly useful way of
looking at the chemical basis of ligand binding
activity, from the viewpoint of the protein molecule.

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As an example of this, we have applied protein signature analysis to one of the Src Homology 3 (SH3) binding domains commonly found in proteins involved in intracellular signal transduction.

5   SH3 domains are small protein modules: polypeptide chains of about 60 amino acid residues that fold to form a unique three dimensional structure, even outside the context of the longer polypeptide chain in the parent protein. It is now

10   well established that SH3 domains mediate protein-protein interactions through the recognition of short proline-rich sequences.

Our goal was to investigate the chemical basis of the interaction between the N-terminal SH3 domain

15   from the cellular adaptor protein, c-Crk (residues 134-191 of the murine sequence), and its target ligand, a proline-rich peptide from the guanine nucleotide exchange protein, C3G. We wanted to change the chemical structure of the SH3 polypeptide chain

20   and observe how this affected the functional properties of the domain.

We decided to introduce a dramatic perturbation in the chemical structure of the protein molecule: deletion of the side chains of two adjacent amino

25   acids in concert with the introduction of an extra backbone methylene. A thioester bond was also introduced to facilitate the identification of protein analogues (see below). The resulting Gly-[COS]-ßAla dipeptide analogue unit can be compared

30   with a native dipeptide sequence (see Figure 22A).

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A   Synthesis of an array of SH3 analogues

We initially focused on a sequence of twenty
amino acids near the middle of the SH3 polypeptide
chain, the region c-Crk(146-165). As a first step an
array of nineteen protein analogues was chemically
synthesized by placing the Gly-[COS]-ßAla dipeptide
unit at each possible dipeptide position within the
twenty amino acid stretch. In this synthesis, we used
the modified stepwise solid phase peptide synthesis
(SPPS) approach that is described in examples 1 and 2
(supra). This method made it possible to prepare all
members of this array of analogues simultaneously in
the course of a single synthesis (see Figure 1). This
modified split-resin procedure ensured that each
individual polypeptide chain in the final product
mixture contained only one analogue unit at a single
defined position. Stepwise synthesis of the full-
length 58 residue SH3 domain polypeptide, with the
introduction of a chemical perturbation at nineteen
defined positions of the polypeptide chain according
to such a split-resin process, gave an array of
analogues as a single product mixture containing the
nineteen desired molecular species.

B.   Functional selection by affinity
       chromatography

The next task was to subject these synthetic
products to functional selection. The N-terminal SH3
domain from c-Crk specifically recognizes a ten
residue proline-rich sequence from the protein, C3G.

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A synthetic peptide containing the proline-rich C3G
sequence was covalently immobilized on commercially
available derivatized agarose beads. In preliminary
experiments, a synthetic SH3 domain corresponding to
5   the wild-type sequence was found to bind specifically
to the C3G peptide affinity column; prolonged washing
with high salt buffer did not elute the synthetic SH3
protein, whereas the use of stronger conditions that
disrupt the specific interactions, in this case 6M
10   guanidine.HCl, led to elution of the protein with
~85% recovery of the applied material. No specific
binding of the synthetic c-Crk SH3 domain to a
control affinity column containing leucine enkephalin
was observed. These procedures have previously been
15   described in example 2 (supra).

Having established the validity of the affinity
column assay, the effects of the dipeptide analogue
units on the binding properties of the SH3 domain
were evaluated. The nineteen member array of
20   synthetic analogues of the 58 residue domain was
passed as one pool over the C3G peptide affinity
column, giving rise to binding and non-binding
populations.

C.   Readout of self-encoded arrays of protein
25   analogues

The third and final step was then to determine
which protein analogues were present in each of the
two pools. The identification of individual molecular
species in a pool of closely related protein
30   analogues is a formidable analytical challenge. One

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way to determine the molecular composition of such a
mixture of protein analogues is to combine mass
spectrometry with the synthetic chemistry approach,
as schematically illustrated in Figure 24.

5   The readout of all members of each pool of
protein analogues was accomplished in a single step
using a chemical decoding approach, similar in
concept to that already described for use with
nucleic acid libraries (supra). A latent readout
10   chemistry was built into each molecule in the course
of the preparation of the protein array by total
chemical synthesis.

         The analogue unit contained a unique thioester
chemical cleavage site which allowed us to
15   chemoselectively 'unzip' (see Figure 22B) the mixture
of analogue polypeptide chains found in a particular
pool, binding or non-binding. When examined by matrix
assisted laser desorption ionization (MALDI) mass
spectrometry (Chait, B.T. & Kent, S.B.H. (1992).
20   Weighing naked proteins: Practical high accuracy mass
measurement of peptides and proteins. *Science* 257,
1885-1894), the resulting sets of 'decoded' peptide
fragments gave characteristic signatures that could
be interpreted as follows. Each component of the mass
25   spectrometric signature reflected the presence of the
corresponding full-length polypeptide chain
(containing the analogue unit) in that pool of intact
protein analogues. Furthermore, the position of the
analogue unit in the original 58 residue c-Crk SH3
30   protein analogue was defined by the position of the

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corresponding signal in the mass spectrometric
signature, as schematically illustrated in Figure 24
(Chait et al. (1993) *Science* **262**, 89-92; Zhao et al.
(1996) *Proc. Natl. Acad. Sci. USA* **93**, 4020-402).

5   E.   Role of SH3 Backbone

        The three components of protein signature
analysis - synthesis, selection, and readout - have
previously been described in detail in a series of
model studies (examples 1 and 2 supra). Here we have
10   applied them to an SH3 domain in order to elucidate
the chemical basis of ligand binding. The results
obtained from applying functional selection/chemical
readout to the 19-member array of protein analogues
corresponding to the N-terminal SH3 domain from c-Crk
15   are shown in Figure 25. The mixture of synthetic
protein analogues was passed over a C3G-peptide
affinity column, to assay for binding activity. The
signature of the parent array of protein analogues
(Figure 25B) is compared with the signature of the
20   pool that showed binding activity (Figure 25C).

        Eight (out of nineteen) members of the array of
protein analogues bound to the C3G peptide. Perhaps
of most interest was the pattern of binding and non-
binding observed for proteins modified within the c-
25   Crk(146-152) region. This sequence of the SH3 protein
corresponds to the so called 'RT loop', a region
known to be involved in ligand binding throughout the
SH3 domain family. The sequence of this part of the c-
Crk SH3 polypeptide is: [-Asn146-Asp-Glu-Glu-Asp-Leu-

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Pro[152]-]. It is evident from the signature of the
functional pool of SH3 analogues (Figure 25C) that
binding to the C3G-derived peptide ligand occurred
even when the side chains of the Asp[147] or Glu[149]
5   residues in the SH3 domain had been removed. Thus,
interaction with the Asp[147] or Glu[149] side chain
carboxyls was <u>not</u> essential for binding (note that
the effect of the replacement of these two residues
on binding <u>specificity</u> cannot be inferred from this
10   experiment). In contrast, removal of the side chain
of Asp[150] by substitution with either the Gly or ßAla
portion of the dipeptide analogue unit virtually
eliminated binding; restoration of Asp[150] restored
binding. It should be noted that Asp[147] and Asp[150] are
15   both conserved in the viral form of the protein, v-
Crk, whereas Glu[149] is replaced with a glycine residue
(Mayer et al. (1993) *J. Virol.* **64**, 3581-3589).

These data are intriguing and offer experimental
support for a difference in the roles of the three
20   acidic side chains in ligand binding, as previously
suggested by the X-ray crystallographic data (Wu, X.,
et al. (1995) *Structure* **3**, 215-226.). As shown in
Figure 31, all three of the side chain carboxylate
functionalities in residues Asp[147], Glu[149], and Asp[150] of
25   the c-Crk SH3 domain make specific interactions with
the side chain -[e]NH$_3$. of Lys[6] in the ligand. From the
protein signature analysis results presented here we
can infer that the primary determinant of binding in
this region of the SH3 molecule is the Asp[150] side
30   chain carboxylate.

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The interaction of Asp[147] and Glu[148] side chains with the ligand peptide may play a different role, perhaps affecting the specificity of binding by discriminating between Lys and Arg side chains at this position. The predominant role of Asp[150] and the different roles of Asp[147] and Glu[148] were both suggested by the crystallography data. In the crystal structure, the $-\epsilon NH_3-$ group of the lysine residue (in the Pro-rich peptide ligand) forms a hydrogen bond to an oxygen atom in the side-chain carboxylate of Asp[150], using the preferred *syn* orientation of the oxygen lone electron pair as shown (Figure 31), whereas the hydrogen bonds to Asp[147] and Glu[148] are in the less favoured *anti* orientation.

The signature analysis data shown in Figure 25C are consistent with this crystallographic observation, because replacement of Asp[150] resulted in gross loss of binding activity, whereas replacement of Asp[147] or Glu[148] did not.

Productive application of the signature analysis approach does not require knowledge of the three dimensional structure of a protein domain. However, the three dimensional structure can be used together with protein signature analysis to give additional insights into the chemical basis of protein function. In the example given here, the combination of signature analysis data with structural studies gave a more informative interpretation of the molecular basis of ligand binding than would have been possible with protein signature data alone. These results also

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show the potential of the protein signature analysis technique to illuminate the chemical reality of mechanisms suggested by the structural data.

F.   Role of SH3 backbone

5   As shown in Figure 25, eight of the 19 protein analogues displayed binding activity, while eleven were inactive. We have discussed the implications of these observations for the roles of specific amino acid side chains (above). How can we relate this data

10   to other aspects of the chemical basis of the binding function of the SH3 domain? The non-functional members of the array of protein analogues could owe their inactivity to any or all of the following factors: deletion of amino acid side chains;

15   insertion of an extra methylene in the polypeptide backbone; or, deletion of the H-bonding ability of the central amide moiety in the analogue structure. Information bearing on these possibilities can be simply obtained by making another array containing

20   alternative analogue structures covering the region of interest in the SH3 domain.

        In this case, we made a second nine-membered array to the region cCrk(156-165) using -Gly-[COS]-Gly- as an analogue unit in which the additional

25   methylene of the original analogue unit was not present (Figure 22A):

        The signature obtained after functional separation, based on binding to the Pro-rich C3G peptide affinity column, and readout of this new

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nine-membered array of protein analogues is shown in
Figure 27. The signature in Figure 27B represents an
expansion of the signature data shown in Figure 25C,
but focussing on the region corresponding to
5   replacement of residues 156-165 of the SH3 domain by
the Gly-[COS]-ßAla analogue. Of the nine Gly-[COS]-
ßAla-containing SH3 analogues in this region, only
four bound to the C3G peptide affinity column. The
other five analogues did not bind under the
10   conditions used. This pattern is identical to that
observed for this region in a data set in which only
these nine anlogues were analysed (example 2; supra).

By contrast, all nine (-Gly-[COS]-Gly-)
containing protein analogues exhibited appreciable
15   binding activity in an identical assay (Figure 27A).
That so many of the protein analogues retained
specific binding activity is a remarkable result,
given the very substantial nature of the chemical
changes made in the polypeptide chain. The data show
20   that neither the pairwise deletion of side chains nor
deletion of the H-bond in the central amide moiety of
the analogue structure was responsible for the lack
of binding exhibited by the five inactive members of
the original -Gly-[COS]-ßAla-containing array of
25   protein analogues covering this region. Rather, it
can be inferred from comparison of the two sets of
data that the observed lack of binding activity was
caused by insertion of the extra methylene in the
polypeptide backbone by the original analogue unit.
30   Thus, it appears that the region defined by residues
c-Crk(156-161) is less tolerant to backbone

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engineering than the region defined by residues c-Crk
(161-165) (Figure 32). The affinity binding assay
used does not discriminate between a gross structural
pertubation and a purely functional effect, since
5   both could result in a loss of activity. However,
none of the amino acids in the region being studied
(residues 156-165) interact directly with the ligand,
suggesting that the observed effects may be
structural in origin.

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MATERIALS AND METHODS

General

Analytical HPLC was performed on a Hewlett-
Packard 1050 system with 214 nm detection using a
5   Vydac C18 column (5 μm, 4.6 x 150 mm) at a flow rate
of 1 mL/min. All runs used a linear 0%-67%B gradient
where buffer A was 0.1% TFA in H_2O and buffer B was
90% acetonitrile, 10% H_2O, 0.09% TFA. Electrospray
mass spectrometric analysis of all synthetic peptides
10   was performed on a Sciex API-III triple quadrupole
electrospray mass spectrometer. Calculated masses
were obtained using the program MacProMass (Sunil
Vemuri and Terry Lee, City of Hope, Duarte, CA);
Buffer B was 90% acetonitrile, 10% H_2O, 0.09% TFA.
15   Semipreparative HPLC was performed on a Rainin HPXL
dual pump system using a Vydac C18 column (10μm, 10 x
250 mm) at 3 mL/min with detection on a Dynamax UV
detector.

Matrix-Assisted Laser Desorption Ionization Mass
20   Spectrometry (MALDI) (example 1):

Mass spectra were recorded using a Vestec Model
VT 2000 laser desorption, linear time-of-flight mass
spectrometer. Samples were desorbed/ionized using
the focused output of a 355 nm frequency tripled
25   Lumonics Model HY 400 Nd:YAG laser (Lumonics, Kanata,
ON, Canada). Ions were accelerated through a dual-
stage source to a total potential of 30 keV and
detected by a 20-stage focused mesh All spectra were
acquired in the positive ion mode and summed over 50

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laser pulses. Time-to-mass conversion was
accomplished by internal calibration using the [M+H]·
and [M+2H]·² ion signals from a standard peptide (MW
2419.1 Da). Samples were prepared by dissolving the
crude peptide array in 1:1 acetonitrile:H_2O, 0.1% TFA
to a concentration of 1-10 μM per peptide component.
2 μL of this solution was mixed with 5 μL of a
saturated solution of 2,5-dihydroxybenzoic acid (DHB)
in the same solvent. Ultimately, 2μL of this mixture
containing ~1-10 pmoles of each peptide component was
added to a stainless steel probe tip (3.14 mm^2.) and
the solvent allowed to evaporate under ambient
conditions.

Solid Phase Peptide Synthesis (example 1).:

Except where noted, all peptides were
synthesized manually according to the *in situ*
neutralization/HBTU activation protocol for Boc solid
phase synthesis as previously described (Schnölzer et
al. *Int. J. Pept. Protein Res.* 1992, *40*, 180-193).
The peptides were synthesized on (4-
Me)benzhydrylamine-copoly(styrene-1% DVB)-resin
(Peninsula Laboratories, 0.93 mmol/g) which after HF
cleavage gives the C-terminal amide. The peptides
were deprotected and cleaved from the resin by
treatment with 10 mL HF, containing 5% anisole, for
one hour at 0°C. After evaporation of the HF, the
crude peptide product was precipitated and washed
with diethyl ether, dissolved in 1:1 Acetonitrile/H_2O
containing 0.1% TFA, and lyophilized.

Synthesis of Boc-Gly-SCH₂CH₂COOH (example 1):

Synthesis of Boc-Gly-SCH$_2$CH$_2$COOH was based on a
previously published procedure (Hojo et al. *Bull.*
Chem. Soc. Jpn. 1991, *64*, 111-117). To a solution of

5   Boc-Gly-OSuc (1.36 g, 5 mmol; Sigma) dissolved in 50
mL CH$_2$Cl$_2$, 3-mercaptopropionic acid (0.5 g, 5 mmol;
Aldrich) and *N,N*-diisopropylethylamine (DIEA; Sigma)
(1.0 g, 7.5 mmol) were added, and the resulting
solution was stirred at room temperature for 15

10   hours. The solvent was reduced by evaporation under
reduced pressure and the resulting oil was dissolved
in ethyl acetate. After two washes with 0.1 M HCl
and four washes with saturated aqueous NaCl, the
ethyl acetate layer was dried over magnesium sulfate.

15   Following concentration, the resulting oil was
dissolved in 40 mL diethyl ether.
Dicyclohexylamine(DCHA) (4.5 mmol, 1 g) was added
dropwise, giving crystals which were recrystallized
from hot ethyl acetate. The DCHA salt was suspended

20   in ethyl acetate and extracted with 0.05 M citric
acid. After three washes with saturated NaCl, the
ethylacetate layer was dried over magnesium sulfate.
After the solution was filtered and concentrated,
trituration with hexane gave Boc-Gly-SCH$_2$CH$_2$COOH as a

25   solid (750 mg, 62 %) FAB MS for C$_{10}$H$_{16}$O$_5$N$_1$S$_1$Na$_1$, MW obsv:
286.0728 Da, calc: 286.0725; melting point 103°-105° C
(104°-106°).

Synthesis of the nine component peptide analogue
array of the parent sequence PFKK-[GDILRIRDKP]-

30   EEAcpRLKLKAR (example 1):

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The sequence EEAcpRLKLKAR was synthesized in
reaction vessel A, Figure 4, on 0.1 mmol MBHA resin.
Onto this sequence, an array of nine Gly-SβAla
substituted peptide analogues was synthesized through
5   the sequence GDILRIRDKP using the protocol described
below. Boc-Gly-SCH$_2$CH$_2$COOH (0.25 mmol, 66 mg) was
preactivated for one hour with DIC (0.25 mmol, 39 μL;
Aldrich) and HOBt (0.25 mmol, 34 mg; Aldrich) in 600
μL DMF (~0.4 M; Aldrich), and used for five
10   consecutive cycles (125 μL/cycle), after which a
second 0.25 mmol of the dipeptide analogue was
activated under the same conditions and used for the
remaining four cycles.

Cycle 1:

15   First, the N$^\alpha$-Boc deprotected peptide-resin (0.10
mmol) was suspended in 10 mL DMF. One milliliter
(~0.01 mmol) of the suspension was removed and added
to a small fritted funnel. This sample was then
neutralized for 1 min with 10% DIEA in DMF, drained,
20   placed in position 1 and reacted with the activated
thioester dipeptide analogue (125 μL, 0.05 mmol).
During neutralization of the sample, the first
subsequent activated amino acid, Boc-proline, was
coupled to the resin in vessel A using manual *in situ*
25   neutralization synthetic cycles with HBTU as the
activating agent. After 20 minutes coupling, the
peptide-resin in vessel A was washed with DMF,
treated with TFA and washed again with DMF. The
first peptide-resin sample was then moved to position
30   1' where dipeptide coupling was allowed to continue.

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Cycle 2:

The deprotected peptide-resin in vessel A was
suspended in 9 mL DMF and 1 mL (~0.01 mmol) was
transferred to a second small fritted funnel and
5   placed in position 1. After neutralization of this
sample, 125 μL (0.05 mmol) of the activated dipeptide
was added to the sample which was placed in the now
open position 1. At the same time, activated Boc-Lys
was added to Vessel A. After the lysine coupling in
10   vessel A was complete, the first (dipeptide analogue)
peptide-resin sample was transferred from position 1'
to reaction vessel B. The peptide-resins in A and B
were then deprotected with TFA, washed and finally
the sample in position 1 was moved to position 1'.

15   This procedure was continued for a total of 10
cycles of chain elongation, with the final cycle
skipping the removal of resin from vessel A.
Finally, the sequence PFKK was added to the peptide-
resin in vessel B, by stepwise SPPS. Following
20   deprotection and cleavage from the resin, the
lyophilized peptide analogue array was analyzed by
analytical reverse phase HPLC, electrospray mass
spectrometry, and MALDI mass spectrometry.

Synthesis of the Thioester-Containing Model Peptide:
25   LYRA-Gly-SβAla-YGGFL·amide (example 1):

YGGFL-4-MeBHA-resin was synthesized on an 0.04
mmol scale using standard manual Boc chemistry
protocols.   Boc-Gly-SβAla (0.2 mmol, 53 mg) and HOBt

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(0.2 mmol, 28 mg) were dissolved in 1mL DMF to which DIC (0.2 mmol, 33 μL) was added. After 30 min, the activated dipeptide was added to the deprotected, neutralized (10% DIEA in DMF, 1 min) YGGFL-MBHA resin
5   and allowed to couple for one hour. The sequence was completed following standard manual cycles to synthesize LYRA-Gly-SßAla-YGGFL-4-MeBHA resin. Following deprotection and cleavage from the resin, the lyophilized peptide was characterized by
10   analytical HPLC and by electrospray mass spectrometry. Observed mass: 1203.5±0.4 Da, calculated mass (average isotope composition): 1203.4 Da.

Cleavage of Thioester-Containing Model Peptide
15   **(example 1):**

The peptide LYRA-Gly-SßAla-YGGFL·amide, was dissolved in 200 μL of 100 mM Tris pH 9.0, 1 M Gn·HCl, vortexed vigorously for 10 seconds and left at 23°C for 30 minutes. No hydrolysis was observed under
20   these conditions. However, addition of 20 μL of 1 M NaOH (to pH~13) gave complete hydrolysis after just 10 minutes. Another sample of the thioester-containing peptide was dissolved in 1 M NH_2OH, 200 mM NH_4HCO_3, pH 6.0 for 30 minutes and completely cleaved
25   into LYRAG-NHOH (observed mass: 593.5±0.5 Da, calculated mass (average isotope composition): 593.7 Da) and $SHCH_2CH_2CO$-YGGFL·amide (observed mass 642.5±0.5 Da, calculated mass (average isotope composition): 642.8 Da).

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Synthesis of Ester-Containing Model Peptide: YKLFAla-[COO]-LeuYGGFL·amide (example 1):

The ester-containing model peptide was
synthesized by a previously established procedure
5   (Bramson et al. *J. Biol.Chem.*1985, *260*, 15452-15457).
2-Hydroxyisocaproic acid ('Leuceic acid') (1.0 mmol,
131 mg) and HOBt (1.1 mmol, 150 mg) were cooled to
0°C in 2 mL 1:1 DMF/CH$_2$Cl$_2$, activated with DIC (1.0
mmol) for 15 min and added to NH$_2$-YGGFL-4-MeBHA resin
10   (0.1 mmol). N-ethylmorpholine (0.25 mmol) was added
and coupling proceeded for 30 min. The ester bond
was created by activating Boc-Ala (1.0 mmol, 190 mg)
with 4-dimethylaminopyridine (0.05 mmol,), DIC (1.0
mmol) and N-ethylmorpholine (0.25 mmol) and reacting
15   with the (α-hydroxy)acyl peptide-resin for 2 hours.
The peptide chain assembly was completed by manual
stepwise SPPS using *in situ* neutralization protocols.
The peptide was then deprotected and cleaved from the
resin and analyzed by analytical HPLC and
20   characterized by electrospray mass spectrometry;
[observed mass: 1291.0±0.5 Da, calculated mass
(average isotope composition): 1290.6 Da].

**Cleavage of the Ester-Containing Model Peptide
(example 1):**

25   The ester-containing peptide YKLFAla-[COO]-
LeuYGGFL·amide was allowed to stand in 6M Guanidine
HCl, 100 mM Na phosphate pH 10 for six hours.
Complete hydrolysis was observed. The resulting
peptides were separated by analytical HPLC and

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characterized by electrospray mass spectrometry.
(YKLFA-OH, [observed mass: 640.5±0.5 Da, calculated
mass (average isotope compositon): 640.8 Da]; HO-
LYGGFL·amide, [observed mass: 669.0±0.5 Da, calculated
5   mass (average isotope compositon): 668.8 Da]. By
contrast, the ester-containing peptide was completely
stable to treatment with 1 M NH$_2$OH, 200 mM NH$_4$HCO$_3$, pH
6.0 for up to 12 hours, as monitored by analytical
HPLC.

10   **Hydroxylamine Cleavage of the Nine Component Peptide
Gly-S&Ala Analogue Array of the Parent Sequence PFKK-
[GDILRIRDKP]-EEAcpRLKLKAR (example 1):**

        0.3 mg (0.1 μmol/component) of the peptide array
was dissolved in 100 μL 1M NH$_2$OH.HCl, 200mM NH$_4$HCO, pH
15   6.0. After 30 minutes, the array was analyzed by
analytical reverse phase HPLC and MALDI mass
spectrometry.

        Solid Phase Peptide Synthesis (example 2)
Except where noted all peptides were synthesized
20   according to the machine-assisted *in situ*
neutralization/HBTU activation protocol for Boc-solid
phase chemistry as previously described (Schnölzer et
al. *Int. J. Pept. Protein Res.* 1992, *40*, 180-193)
using a modified Applied Biosystems 430A peptide
25   synthesizer. Following synthesis, the N·Boc group was
removed, and the peptide cleaved from the resin with
simultaneous removal of sidechain protecting groups
by treatment for 1 hour at 0°C with anhydrous HF
containing 5% anisole or 5% p-cresol as a scavenger.

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After evaporation of the HF, the crude peptide was precipitated and washed with cold diethyl ether, dissolved in 1:1 acetonitrile:H_2O containing 0.1% TFA, filtered to remove the resin, and lyophilized.

5   **Synthesis of native N-terminal cCrk SH3 domain (cCrk residues 134-191) (example 2):**

The 58 amino acid residue polypeptide was synthesized using 0.12 mmol Boc-Arg(Tos)-OCH$_2$-Pam resin, loading 0.59 mmol/g (Applied Biosystems,
10   Foster City, CA). Standard sidechain protecting groups (Schnölzer et al. *Int. J. Pept. Protein Res.* 1992, *40*, 180-193) were used except for the tryptophan indole moiety which was left unprotected because subsequent syntheses of base labile analogues
15   would not permit the nucleophilic removal of the usual formyl protecting group. HF cleavage of 300 mg of peptide resin from the resin gave 195 mg of lyophilized crude peptide. A 5 mg sample of the crude peptide was purified by semipreparative HPLC on
20   a 20%-40%B gradient over 45 minutes to give 1.4 mg purified product (23% yield, calculated from the original loading of the resin). The purified peptide product was a single peak by analytical HPLC and was pure by electrospray mass spectrometry: Observed mass
25   6962±1 Da: Calculated mass for $C_{313}H_{470}N_{84}O_{93}S_1$ 6961.8 Da (average isotope distribution).

Functional characterization of the synthetic 58-residue cCrk N-terminal SH3 domain:

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The affinity of the synthetic SH3 domain to two
C3G derived peptides was determined by measuring the
increase in the protein domain tryptophan
fluorescence upon ligand binding, following the
5   procedure described in Lim et al. *Protein Science*.
1994, *3*, 1261-1266. The purified and lyophilized 58
residue polypeptide chain (0.2 mg) was folded by
dissolving in 0.6 mL of 20 mM Hepes, 60 mM NaCl, pH
7.3 to produce a folded protein solution (~50 μM).
10   Peptide stock solutions of both the C3G-derived
peptide [PPPALPPKKR.amide] (71.9 μM) and the C3G-
derived peptide designed for attachment to an
affinity column [Ac-CWAcp-PPPALPPKKR.amide] (71.5 μM)
were obtained by dissolving the lyophilized peptides
15   in the same buffer. Peptide concentrations were
determined by quantitative amino acid analysis.

**Synthesis of the nine component analogue array of
cCrk   (134-191) (example 2):**

The target array of analogue polypeptide chains
20   is shown in **Figure 23**. The first part of the
sequence, corresponding to cCrk (166-191), was
synthesized on a 0.2 mmol Boc-Arg(Tos)-OCH$_2$-Pam resin,
(0.59 mmol/g) using machine-assisted synthetic
cycles. The array of polypeptide analogues was
25   manually synthesized on ~0.05 mmol (250 mg) of this
peptide resin by a modified split-resin procedure
previously described in Hayashibara et al. *J. Am.
Chem. Soc.* **1991**, *113*, 5103-5106 (similar readout
systemdeveloped for use in DNA systems). Boc-Gly-
30   SßAla-OH (0.25 mmol, 66 mg) was preactivated for one

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hour with DIC (0.25 mmol, 38 μL; Aldrich) and HOBt
(0.25 mmol; Aldrich) in DMF: total volume 650 μL.
The apparatus for manual synthesis consisted of two
standard manual synthesis reaction vessels, labeled A
5   and B and two small fritted funnels in a test tube
rack in positions 1 and 1'.

Cycle 1:

First, the N°-Boc-deprotected cCrk(166-
191)peptide-resin (50 μmol) was suspended in 10 mL
10   DMF. One milliliter (~5 μmol) of the suspension was
removed and added to a small fritted funnel. This
sample was then neutralized for 1 min with 10% DIEA
in DMF, drained, placed in position 1 and reacted
with the activated dipeptide analogue (65 μL, 25
15   μmol). During neutralization of the sample, the
coupling of the first activated amino acid, Boc-Pro[145],
to the peptide-resin in reaction vessel A was
initiated using manual *in situ* neutralization
synthetic cycles with HBTU as the activating agent.
20   After 20 minutes, vessel A was washed with DMF,
treated with TFA and washed again with DMF. The
removed peptide-resin sample in position 1 was then
moved to position 1' where coupling of the dipeptide
analogue was continued.

25   Cycle 2:

The peptide-resin in vessel A was suspended in 9
mL DMF and 1 mL (~5 μmol) was transferred to a second
small fritted funnel and placed in position 1. After

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neutralization of this sample and addition of
activated Lys¹⁵⁴ to A, the activated dipeptide (65 µL,
25 µmol) was added to the sample which was placed in
the now open position 1. Following the Boc-Lys¹⁵⁴
5   coupling in vessel A, the first analogue-modified
peptide resin sample in position 1' was washed with
DMF and then transferred to reaction vessel B. The
peptide-resins in reaction vessels A and B were then
deprotected with TFA, washed and finally, the second
10   peptide-resin sample in position 1 was moved to
position 1', where coupling of the dipeptide analogue
was continued.

        The procedure described above was continued for
10 cycles, through the addition of residue 156. In
15   the final cycle the removal of a peptide-resin sample
from vessel A and dipeptide coupling steps were
omitted. Finally, half of the mixture of peptide-
resins in vessel B (25 µmol total) containing nine
peptide analogues, was removed and transferred to an
20   Applied Biosystems 430A peptide synthesizer for
addition of the remaining amino acids, cCrk 134-155.
This procedure gave a product mixture of 58 residue
peptide-resins. Following deprotection and cleavage
from the resin, the lyophilized peptide array was
25   analyzed by analytical reverse phase HPLC and MALDI
mass spectrometry.

**Synthesis of C3G-derived ligand and control peptides
(example 2):**

        The peptides corresponding to the C3G-derived

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ligand, Ac-CWAcp-PPPALPPKKR·amide and the control, Ac-CAcpYGGFL·amide, were synthesized on 4-methyl benzhydrylamine resin (0.93 mmol/g Peninsula Laboratories) and were cleaved from the resin support
5   using p-cresol as a scavenger, and then purified by semipreparative HPLC using a 25%-50% acetonitrile gradient over 30 minutes. The products were characterized by ESMS. Ac-CWAcpPPPALPPKKR·amide; Observed mass: 1543±1 Da. Calculated mass for
10   $C_7H_{11}N_{20}O_{14}S_1$ (average isotope composition): 1543.9 Da, Ac-CAcpYGGFL·amide; Observed mass: 813.5±0.5 Da, Calculated mass for $C_3H_{56}N_6O_9S_1$ (average isotope composition): 814.0 Da.

Preparation of affinity columns (example 2):

15   The C3G-derived synthetic peptide affinity column was prepared by adding 10 mg of lyophilized Ac-CWAcpPPPALPPKKR·amide (Acp = e-amino caproic acid) in 2 mL of 50 mM Tris, 5 mM EDTA, pH 8.0 buffer to Sulfolink™ resin (Pierce), equilibrated in the same
20   buffer, for 1 hour while shaking. Unreacted iodoalkyl groups on the resin were then blocked by treatment with 50 mM cystamine, 50 mM Tris, 5 mM EDTA, pH 8.0 buffer for 1 hour. The loading of the column was determined by UV absorbance of the unreacted peptide
25   solution and was approximately 5 μmol/mL. A similar procedure was used to attach the control peptide Ac-CAcpYGGFL·amide to the another batch of the Sulfolink™ support.

Affinity selection of synthetic cCrk SH3 domain

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(example 2):

Lyophilized crude synthetic polypeptide
corresponding to the cCrk SH3 domain (1.5 mg) and BSA
(8.5 mg) were dissolved in 20 mM HEPES, 50 mM NaCl,
pH 7.3 buffer (400 μL) and loaded on to a 1 mL C3G-
derived synthetic peptide affinity column pre-
equilibrated with the same buffer. The column was
washed with 4 mL each of 50 mM NaCl, 100 mM NaCl, 200
mM NaCl, 300 mM NaCl, 400 mM NaCl, 500 mM NaCl and
1000 mM NaCl, 0.1 M phosphate, pH 7.0 buffer. The
column was then washed with 1 M NH_2OH 200 mM NH_4CO_3 pH
6.0 (4 mL) and finally eluted in 6M Gn.HCl 100 mM
phosphate pH 6.5 (4 mL). Samples from all column
fractions were monitored by absorbance at 280 nm and
by HPLC.

**Affinity chromatography the nine-membered arrays
(example 2):**

Affinity chromatography of the arrays of protein
analogues was carried out as follows. The crude
protein array (1.5 mg) was dissolved in 20 mM HEPES,
50 mM NaCl, pH 7.3 buffer (600 μL) and loaded on to a
1 mL C3G-derived synthetic peptide affinity column
pre-equilibrated with the same buffer. After
incubation at room temperature for a period of 6-8
hours the column was washed with 0.5 M NaCl in 0.1 M
sodium phosphate, pH 7.0 buffer (6 x 1 mL) to remove
any non-specifically bound proteins. The first two 1
mL fractions were collected and immediately mixed
with an equal volume of 1 M NH_2OH, 20 mM NH_4HCO_3, pH

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5.5 buffer to cleave any thioester-containing protein
analogues. Following a second 6 x 1 mL column wash
with 1 M NaCl in 0.1 M sodium phosphate, pH 7.0
buffer, specifically-bound protein analogues were
5   chemically cleaved and eluted from the affinity
column by washing with 1 M NH$_2$OH, 20 mM NH$_4$HCO$_3$, pH 5.5
buffer (4 x 1 mL). Samples of the eluted fractions
were monitored by UV absorbance at 280 nm. This
procedure was used for both the C3G peptide column
10   and for the control column loaded with Ac-C-Acp-
YGGFL·amide. To accommodate MALDI analysis, both the
0.5M NaCl wash and 1 M NH$_2$OH fractions were desalted
on a low pressure, disposable C-18 column, washed
with HPLC buffer A and peptide fragments eluted with
15   1.5 mL 60% acetonitrile in water, 0.1% TFA.

**MALDI mass spectrometric analysis of peptide
fragments (example 2):**

After desalting, the affinity column fractions
were analyzed by MALDI mass spectrometry . Samples
20   were prepared by adding a 2 μL aliquot of the 1.5 mL
desalted column fraction to 5 μL of a saturated
solution of α-cyano-4-hydroxycinnamic acid in 50 %
acetonitrile in water, 0.1% TFA. From this mixture,
2 μL, containing ~1-10 pmole of each peptide
25   component was added to a stainless steel probe tip
(3.14 mm^2.) and the solvent allowed to evaporate
slowly under ambient conditions. Mass spectra were
recorded using a prototype laser desorption, linear
time-of-flight mass spectrometer from Ciphergen
30   Biosystems Inc. (Palo Alto, CA). Samples were

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ionized using 337 nm radiation output from a nitrogen
laser (Laser Science, Inc., Newton MA). All spectra
were acquired in the positive ion mode and summed
over 20-50 laser pulses. Time-to mass conversion was
5   accomplished by internal calibration using the [M+H]$^+$
signals from the largest and smallest peptide
components in each array.

Synthesis of Peptides (example 3):

With the exception of protein arrays all
10   peptides were chemically synthesized according to
optimized solid-phase methods (Schnölzer (1992) *Int.
J. Pept. Protein Res.* **40**, 180-193) and purified by
preparative reverse-phase HPLC using a Vydac C-18
column. In all cases, peptide composition and purity
15   were confirmed by electrospray mass specrometry and
analytical reverse-phase HPLC.

Synthesis of C-Crk SH3 Protein Arrays (example 3):

A detailed description of the split-resin
procedure used (examples 1 and 2; supra). Briefly,
20   the technique involves the use of two reaction
vessels with identical synthetic manipulations being
carried out in each. Standard stepwise chain assembly
was initiated on resin in the first vessel (0.2 mmole
scale); peptide-resin samples were repeatedly removed
25   from the first vessel at each stage of the synthesis,
and analogue units were introduced into the
polypeptide chain by coupling as preformed HOBt
esters; after modification, the samples were
transferred to the second vessel for completion of

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the chain assembly by standard stepwise chain
assembly. The size of the samples was adjusted to
yield approximately equal molar amounts of each
protein analogue in the array (dependent on the
5   number af protein analogues in a given array). The
dipeptide analogues Gly-[COS]-ßAla and Gly-[COS]-Gly
were prepared as previously described (Hojo et
al.(1991) *Bull. Chem. Soc. Jpn.* 64, 111-117). Upon
completion of the synthesis, each parent protein
10   array was characterized as follows: crude protein
array (~1 mg) was dissolved in a cleavage buffer
consisting of 1 M NH_2OH, 20 mM NH_4HCO_3, pH 6.5 buffer
(1 ml) and stirred for 15 minutes. The cleaved arrays
were then exchanged into a 70% CH_3CN:30% H_2O, 0.1% TFA
15   solvent system (using a 1 ml C-18 desalting column)
and immediately analysed by MALDI mass spectrometry.

Synthesis of Peptide Affinity Columns (example 3):

The C3G peptide affinity column was prepared as
follows. The peptide Ac-CWBPPPALPPKKR.amide (B = ε-
20   aminocaproic acid) was dissolved in 50 mM Tris, 5 mM
EDTA, pH 8.0 (10 mg in 2 ml) and shaken with
Sulfolink™ resin (Pierce) for 1 hour. Unreacted
iodoalkyl groups on the resin were then blocked by
treatment with 50 mM cystamine, 50 mM Tris, 5 mM
25   EDTA, pH 8.0 buffer. The loading of the column was
determined by UV to be approximately 5 μmole/ml. A
similar procedure was used to attach the control
peptide Ac-CBYGGFL.amide (YGGFL = leucine enkephalin)
to the Sulfolink™ support.

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Affinity Selection of Synthetic c-Crk SH3 (example 3):

HPLC purified synthetic c-Crk SH3 (1.5 mg) was
dissolved in 20 mM Hepes, 50 mM NaCl, pH 7.3 buffer
5   (400μl) and applied to a 1 mL C3G peptide affinity
column pre-equilibrated with the same buffer. After
6-8 hours (required for optimal binding) the column
was washed with, in turn: 0.5 M NaCl, 0.1 M sodium
phosphate, pH 7.0 buffer (6 x 1 ml), 1 M NaCl, 0.1 M
10   sodium phosphate, pH 7.0 buffer (6 x 1 ml) and 1 M.
NH_2OH, 20 mM NH_4HCO_3, pH 5.5 buffer (6 x 1 ml). The
applied material did not elute from the column under
any of these conditions, but was readily recovered
(with ~85% yield) by washing the column with a 6 M
15   GuHCl, 0.1 M sodium phosphate, pH 7.0 buffer (2 x 1
ml). In contrast, the synthetic SH3 domain did not
specifically bind to the leucine enkaphalin control
column under identical conditions to the above.

Affinity Selection of Protein Arrays (example 3):

20   The crude protein array (1.5 mg) was dissolved
in 20 mM Hepes, 50 mM NaCl. pH 7.3 buffer (600μl) and
loaded on to a 1 ml C3G peptide affinity column pre-
equilibrated with the same buffer. After 6-8 hours
the non-specifically bound material was eluted from
25   the column by washing with 0.5 M NaCl, 0.1 M sodium
phosphate, pH 7.0 buffer (6 x 1 ml). Eluted material
(typically in the first and second wash) was
immediately cleaved by dilution into 1 M NH_2OH, 20 mM
NH_4HCO_3, pH 5.5 buffer. Following further column

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washing with 1 M NaCl, 0.1 M sodium phosphate, pH 7.0
buffer, the specifically bound material (active pool)
was chemically cleaved and simultaneously eluted from
the affinity column by washing with 1 M NH_2OH, 20 mM

5   NH_4HCO_3, pH 6.5 buffer (4 x 1 ml). Eluted fractions
were exchanged into a 70% CH_3CN:30% H_2O, 0.1% TFA
solvent system and immediately analysed by MALDI mass
spectrometry.

MALDI Analysis of Peptide Arrays (example 3):

10   All samples were prepared by adding 2 μL of the
desalted column fraction to 5 μL of a saturated
solution of α-cyano cinnaminic acid in 50 %
acetonitrile in water, 0.1% TFA. From this mixture,
2μL, containing ~1-10 pmole of each pepide component

15   was added to a stainless steel probe tip and the
solvent allowed to evaporate under ambient
conditions. Mass spectra were recorded using a
prototype laser desorption, linear time-of-flight
mass spectrometer from Ciphergen Biosystems (Palo

20   Alto, CA). Samples were desorbed/ionized using 337 nm
radiation output from a nitrogen laser (Laser
Science, Inc., Newton MA). All spectra were acquired
in the positive ion mode and summed over 20-50 laser
pulses. Time-to mass conversion was accomplished by

25   internal calibration using the the $[M+H]^+$ signals from
the largest and smallest peptide components in each
array.

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What is claimed is:

1. A method for obtaining a molecular signature of a
protein, the protein having an amino acid sequence
with length m, each amino acid position within the
sequence being represented by $(aa)_n$ where $1 \leq n \leq m$, the
protein having a binding affinity with respect to a
target molecule under binding conditions, the
molecular signature being defined by a subsequence of
the amino acid sequence selected from amongst
positions $(aa)_n$ which, if individually replaced by a
substitute amino acid, lead to a loss of binding
affinity by the protein with respect to the target
molecule, the method comprising the following steps:

Step A: providing a peptide ladder library with m
        peptides, each peptide being represented by
        $(peptide)_n$, where $1 \leq n \leq m$, each peptide having the
        same amino acid sequence as the protein except
        that position $(aa)_n$ of $(peptide)_n$ is replaced by
        the substitute amino acid, the substitute amino
        acid at position $(aa)_n$ being linked to the amino
        acid at position $(aa)_{n+1}$ by means of a labile
        bond; then

Step B: contacting the peptide ladder library of
        said step A with the target molecule under
        binding conditions for forming bound peptides
        and unbound peptides, the bound peptides being
        bound to the target molecule; then

Step C: separating the unbound peptides from the
        bound peptides of said Step B for obtaining

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separated unbound peptides, each separated
unbound peptide having the substitute amino acid
only at positions $(aa)_n$ corresponding to the
subsequence which defines the molecular
5   signature of the protein with respect to the
target molecule; then

Step D: cleaving the labile bond of the separated
unbound peptides obtained in said Step C for
producing peptide cleavage products, each
10   peptide cleave product corresponding to one of
the positions $(aa)_n$ from the subsequence which
defines the molecular signature; then

Step E: detecting and identifying each of the
peptide cleavage products of said Step D; and
15   then

Step F: constructing the subsequence which defines
the molecular signature of the protein with
respect to the target molecule using the
identity of the peptide cleavage products of
20   said Step E.

2. A method for obtaining a molecular signature of a
protein as described in Claim 1 wherein the labile
bond within the peptide of said Step A is selected
25   from the group consisting of thioester bonds and
ester bonds.

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3. A method for obtaining a molecular signature of a
protein as described in Claim 1 wherein the
substitute amino acid in said Step A is selected from
the group consisting of L-alanine, L-arginine, L-
5   aspartic acid, L-asparagine, L-cysteine, L-cystine,
L-glutamic acid, L-glutamine, L-glycine, L-histidine,
L-isoleucine, L-leucine, L-lysine, L-methionine, L-
phenylalanine, L-proline, L-serine, L-threonine, L-
tryptophan, L-tyrosine, L-valine, D-alanine, D-
10   arginine, D-aspartic acid, D-asparagine, D-cysteine,
D-cystine, D-glutamic acid, D-glutamine, D-glycine,
D-histidine, D-isoleucine, D-leucine, D-lysine, D-
methionine, D-phenylalanine, D-proline, D-serine, D-
threonine, D-tryptophan, D-tyrosine, D-valine, L-α-
15   aminobutyric acid, D-α-aminobutyric acid, L-
γ-aminobutyric acid, D-γ-aminobutyric acid, L-
ε-aminocaproic acid, D-ε-aminocaproic acid, L-
homophenylalanine, D-homophenylalanine, L-
alloisoleucine, D-alloisoleucine, L-β-2-
20   napthylalanine, D-β-2-napthylalanine, L-norvaline, D-
norvaline, L-ornithine, D-ornithine, L-pyridyl
alanine, D-pyridyl alanine, L-2-thienylalanine, D-2-
thienylalanine L-methyltyrosine, D-methyltyrosine, L-
citrulline, D-citrulline, L-homocitrulline, and D-
25   homocitrulline.

4. A method for obtaining a molecular signature of a
protein as described in Claim 1 wherein, in said Step
A, the substitute amino acid at position $(aa)_n$ is
30   attached to a second substitute amino acid to form a

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footprint of two substitute amino acids.

5. A method for obtaining a molecular signature of a
protein as described in Claim 1 wherein, in said Step
5   A, the substitute amino acid at position $(aa)_n$ is
attached to a second and a third substitute amino
acid to form a footprint of three substitute amino
acids.

10   6. A method for obtaining a molecular signature of a
protein, the protein having an amino acid sequence
with length m, each amino acid position within the
sequence being represented by $(aa)_n$ where $1 \leq n \leq m$, the
protein having a binding affinity with respect to a
15   target molecule under binding conditions, the
molecular signature being defined by a subsequence of
the amino acid sequence selected from amongst
positions $(aa)_n$ which, if individually replaced by a
substitute amino acid, lead to a loss of binding
20   affinity by the protein with respect to the target
molecule, the method comprising the following steps:

    Step A: providing a peptide ladder library with m
        peptides, each peptide being represented by
        $(peptide)_n$, where $1 \leq n \leq m$, each peptide having the
25   same amino acid sequence as the protein except
        that position $(aa)_n$ of $(peptide)_n$ is replaced by
        the substitute amino acid, the substitute amino
        acid at position $(aa)_n$ being linked to the

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amino acid at position $(aa)_{n+1}$ by means of a
labile bond; then

Step B: contacting the peptide ladder library of
said step A with the target molecule under
5   binding conditions for forming bound peptides
and unbound peptides, the bound peptides being
bound to the target molecule; then

Step C: separating the unbound peptides from the
bound peptides of said Step B for obtaining
10   separated bound peptides, each separated bound
peptide lacking any substitute amino acid at
position $(aa)_n$ corresponding to the subsequence
which defines the molecular signature of the
protein with respect to the target molecule;
15   then

Step D: cleaving the labile bond of the separated
bound peptides obtained in said Step C for
producing peptide cleavage products, each
peptide cleave product corresponding to one of
20   the positions $(aa)_n$ not included within the
subsequence which defines the molecular
signature; then

Step E: detecting and identifying each of the
peptide cleavage products of said Step D; and
25   then

Step F: constructing the subsequence which defines
the molecuclar signature of the protein with
respect to the target molecule using the

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identity of the peptide cleavage products of
said Step E.

7. A method for obtaining a molecular signature of a
protein as described in Claim 6 wherein the labile
bond within the peptide of said Step A is selected
from the group consisting of thioester bonds and
ester bonds.

8. A method for obtaining a molecular signature of a
protein as described in Claim 6 wherein the
substitute amino acid in said Step A is selected from
the group consisting of L-alanine, L-arginine, L-
aspartic acid, L-asparagine, L-cysteine, L-cystine,
L-glutamic acid, L-glutamine, L-glycine, L-histidine,
L-isoleucine, L-leucine, L-lysine, L-methionine, L-
phenylalanine, L-proline, L-serine, L-threonine, L-
tryptophan, L-tyrosine, L-valine, D-alanine, D-
arginine, D-aspartic acid, D-asparagine, D-cysteine,
D-cystine, D-glutamic acid, D-glutamine, D-glycine,
D-histidine, D-isoleucine, D-leucine, D-lysine, D-
methionine, D-phenylalanine, D-proline, D-serine, D-
threonine, D-tryptophan, D-tyrosine, D-valine, L-α-
aminobutyric acid, D-α-aminobutyric acid, L-
γ-aminobutyric acid, D-γ-aminobutyric acid, L-
ε-aminocaproic acid, D-ε-aminocaproic acid, L-
homophenylalanine, D-homophenylalanine, L-
alloisoleucine, D-alloisoleucine, L-β-2-
napthylalanine, D-β-2-napthylalanine, L-norvaline, D-

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norvaline, L-ornithine, D-ornithine, L-pyridyl
alanine, D-pyridyl alanine, L-2-thienylalanine, D-2-
thienylalanine L-methyltyrosine, D-methyltyrosine, L-
citrulline, D-citrulline, L-homocitrulline, and D-
5   homocitrulline.

9. A method for obtaining a molecular signature of a
protein as described in Claim 6 wherein, in said Step
A, the substitute amino acid at position $(aa)_n$ is
10   attached to a second substitute amino acid to form a
footprint of two substitute amino acids.

10. A method for obtaining a molecular signature of a
protein as described in Claim 6 wherein, in said Step
15   A, the substitute amino acid at position $(aa)_n$ is
attached to a second and a third substitute amino
acid to form a footprint of three substitute amino
acids.

20   11. A peptide ladder library corresponding to a
protein having an amino acid sequence with length m,
each amino acid position within the sequence of the
protein being represented by $(aa)_n$ where $1 \leq n \leq m$, the
peptide ladder library comprising:

25   m peptides, each peptide being represented by
$(peptide)_n$, where $1 \leq n \leq m$, each peptide having the same

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amino acid sequence as the protein except that
position $(aa)_n$ of $(peptide)_n$ is replaced by a
substitute amino acid, the substitute amino acid at
position $(aa)_n$ being linked to the amino acid at
5   position $(aa)_{n+1}$ by means of a labile bond.

12. A peptide ladder library as described in Claim 11
wherein the labile bond within the peptide is
selected from the group consisting of thioester bonds
10   and ester bonds.

13. A peptide ladder library as described in Claim 11
wherein the substitute amino acid is selected from
the group consisting of L-alanine, L-arginine, L-
15   aspartic acid, L-asparagine, L-cysteine, L-cystine,
L-glutamic acid, L-glutamine, L-glycine, L-histidine,
L-isoleucine, L-leucine, L-lysine, L-methionine, L-
phenylalanine, L-proline, L-serine, L-threonine, L-
tryptophan, L-tyrosine, L-valine, D-alanine, D-
20   arginine, D-aspartic acid, D-asparagine, D-cysteine,
D-cystine, D-glutamic acid, D-glutamine, D-glycine,
D-histidine, D-isoleucine, D-leucine, D-lysine, D-
methionine, D-phenylalanine, D-proline, D-serine, D-
threonine, D-tryptophan, D-tyrosine, D-valine, L-α-
25   aminobutyric acid, D-α-aminobutyric acid, L-
γ-aminobutyric acid, D-γ-aminobutyric acid, L-
ε-aminocaproic acid, D-ε-aminocaproic acid, L-
homophenylalanine, D-homophenylalanine, L-
alloisoleucine, D-alloisoleucine, L-β-2-

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napthylalanine, D-β-2-napthylalanine, L-norvaline, D-
norvaline, L-ornithine, D-ornithine, L-pyridyl
alanine, D-pyridyl alanine, L-2-thienylalanine, D-2-
thienylalanine L-methyltyrosine, D-methyltyrosine, L-
5   citrulline, D-citrulline, L-homocitrulline, and D-
homocitrulline.

14. A peptide ladder library as described in Claim 11
wherein the substitute amino acid at position $(aa)_n$
10   is attached to a second substitute amino acid to form
a footprint of two substitute amino acids.

15. A peptide ladder library as described in Claim 11
wherein the substitute amino acid at position $(aa)_n$
15   is attached to a second and a third substitute amino
acid to form a footprint of three substitute amino
acids.

16. A method for constructing a peptide ladder
20   library corresponding to a protein having an amino
acid sequence with length m, each amino acid position
of the protein being represented by $(aa)_n$ where
$1 \leq n \leq m$, the peptide library including m peptides, each
peptide being represented by $(peptide)_n$, where $1 \leq n \leq m$,
25   each peptide having the same amino acid sequence as
the protein except that position $(aa)_n$ of $(peptide)_n$
is replaced by a substitute amino acid, the

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substitute amino acid at position $(aa)_n$ being linked
to the amino acid at position $(aa)_{n+1}$ by means of a
labile bond, the method comprising the following
steps:

5   Step A: providing a first reaction vessel
            containing a first pool of nascent peptides
            having a length of m-n and the amino acid
            sequence between n+1 and m of the protein, the
            nascent peptides being attached to a matrix
10   material;

        Step B: providing a second reaction vessel with a
            first pool of nascent ladder peptides having a
            length of m-n and the amino acid sequence
            between n+1 and m of the protein except that
15   each (nascent ladder peptide)$_p$ has the
            substitute amino acid at position $(aa)_p$, where
            $n+1 \leq p \leq m$, the nascent ladder peptides being
            attached to a matrix material; then

        Step C: transferring an aliquot of matrix material
20   from the first reaction vessel to a third
            reaction vessel; then

        Step D: elongating the first pool of nascent
            peptides in the first reaction vessel by
            addition of the amino acid of position $(aa)_n$ to
25   form a second pool of nascent peptides having a
            length of m-n+1;

        Step E: elongating the aliquot of nascent peptides
            in the third reaction vessel by addition of the

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substitute amino acid of position (aa)$_n$ by
means of labile bond to form a nascent ladder
(peptide)$_n$ having a length of m-n+1;

Step D: elongating the first pool of nascent
5   peptide ladders in the third reaction vessel by
addition of the amino acid of position (aa)$_n$ to
form a partial second pool of nascent peptide
ladders having a length of m-n+1; then

Step F: transferring the product of the third
10   reaction vessel in said Step E to the second
reaction vessel to complete the second pool of
nascent peptide ladders having a length of m-
n+1; and then

Step G: repeating said Steps C through F until n=1
15   and the second reaction vessel contains the
peptide ladder library.

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FIGURE 1

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Step 1: Synthesis of polypeptide family.
Synthetic marker scanned through target polypeptide
sequence. See figure 1 for details.

Step 2: Selection of polypeptide family.
Mixture can be screened for some structural
of functional property. For example, affinity
chromatography could be used to separate
binding from non-binding components of the
polypeptide family.

1. Bind

2. Wash

3. Elute

Step 3: Chemical cleavage and analysis of fragments.
Following selection, the synthetic marker
is selectively cleaved with say a nucleophile,
and the resulting peptide fragments analysed
by HPLC and mass spectrometry. Once
identified these fragments allow the original
location of the marker within the parent
molecule to be determined.

Inactive

Active

1. Cleavage

2. Analysis

FIGURE 2

FIGURE 3

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Vessel A   Vessel B

FIGURE 4

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FOLD

FIGURE 5

SCREENING | FUNCTIONAL
PROCESS | SELECTION

BINDERS   NON BINDERS

FIGURE 6

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SCREENED FAMILY OF PEPTIDES EITHER BINDERS OR NONBINDERS
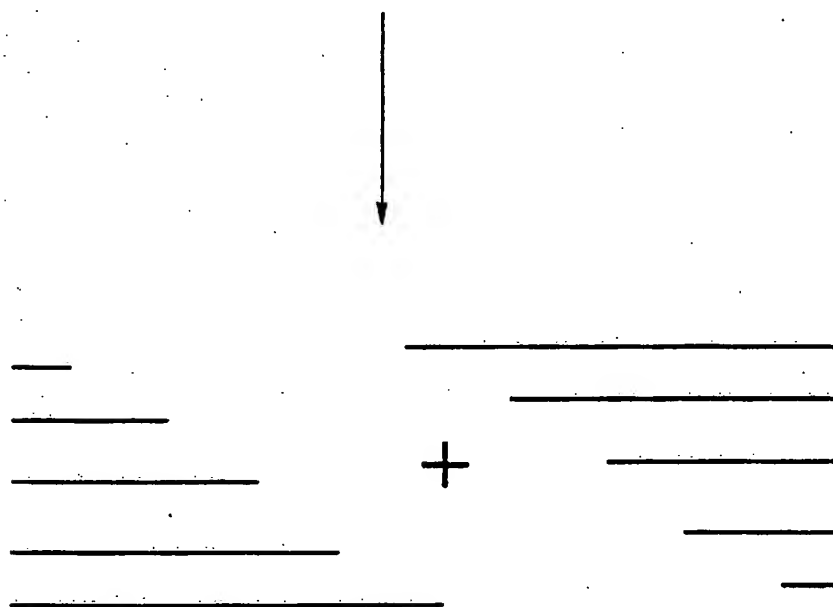
FIGURE 7

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FIGURE 8

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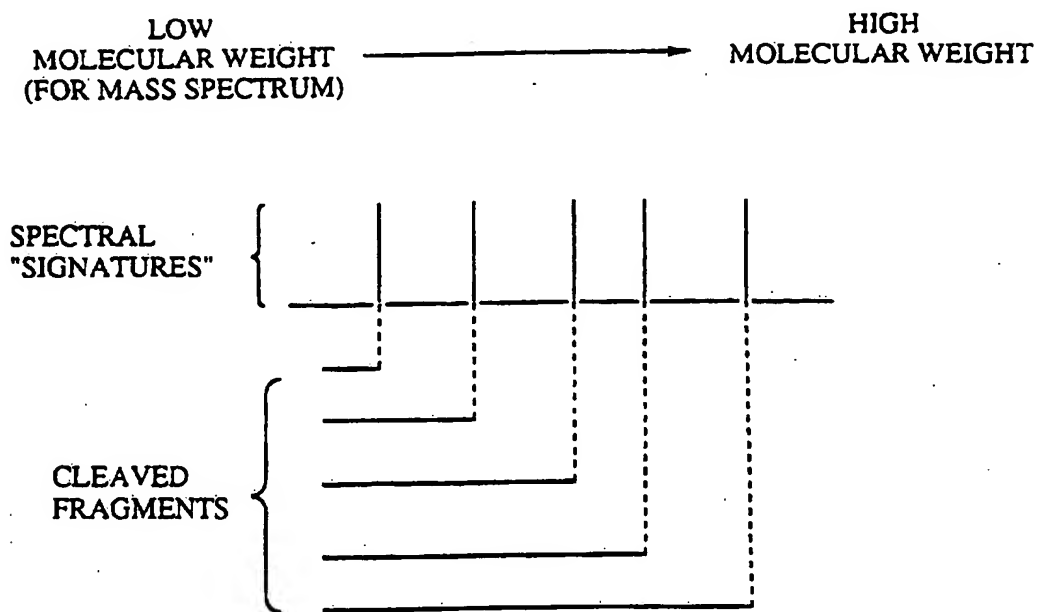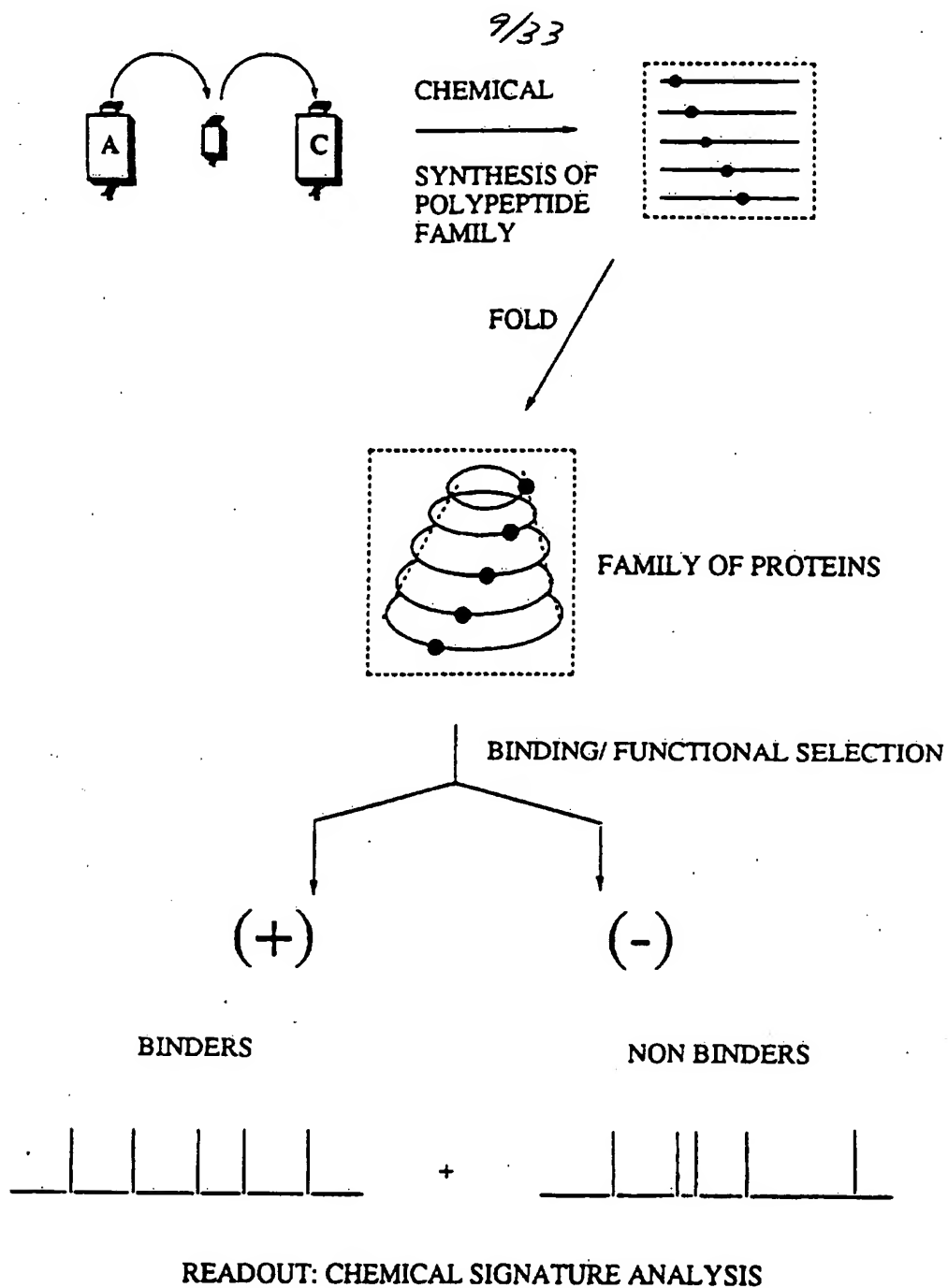
FIGURE 9

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DESIGN
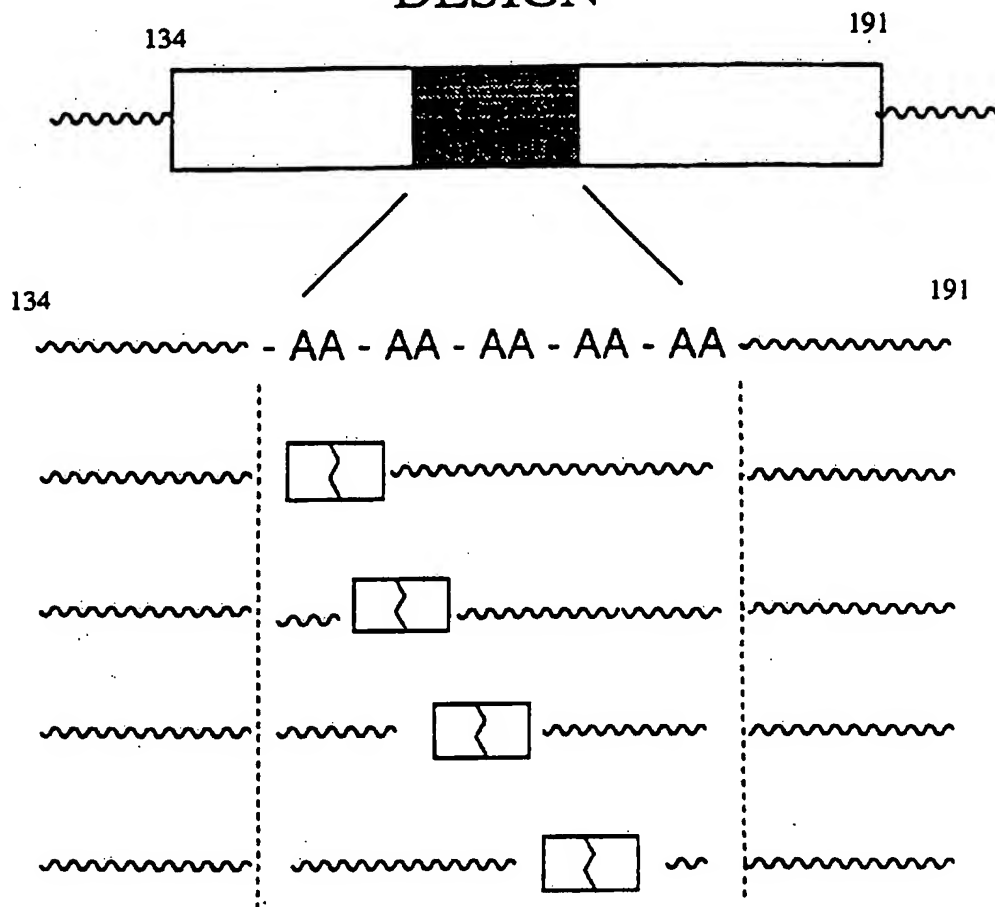
FIGURE 10

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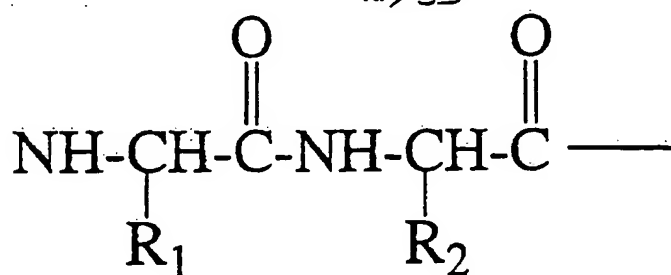
NATIVE
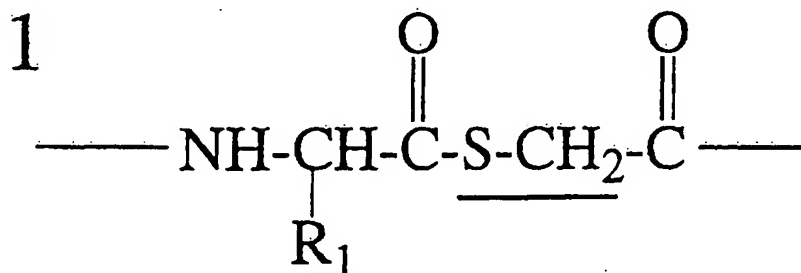
1   DELETE
                                               1 SIDECHAIN
                                               1 N-H
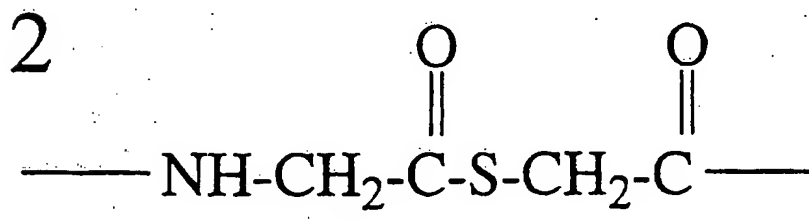
2   DELETE
                                               2 SIDECHAIN
                                               1 N-H
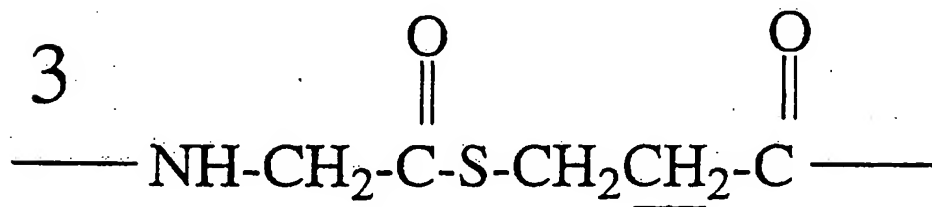
3   DELETE
                                               2 SIDECHAIN
                                               1 N-H
                                               1 CH$_2$ IN
                                               BACKBONE

FIGURE 11

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$$1 \quad \underset{\displaystyle -\!\!-\!C\text{-}NH\!-\!\!-}{\overset{\displaystyle O}{\|}}$$

$$2 \quad \underset{\displaystyle -\!\!-\!C\text{-}O\!-\!\!-}{\overset{\displaystyle O}{\|}}$$

$$3 \quad \underset{\displaystyle -\!\!-\!C\text{-}S\!-\!\!-}{\overset{\displaystyle O}{\|}}$$

$$4 \quad \underset{\displaystyle -\!\!-\!C\text{-}NH\text{-}O\!-\!\!-}{\overset{\displaystyle O}{\|}}$$

FIGURE 12

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ITERATION:

SEQUENCE   SCAN A
INFO. ———→   PERTURBATION ———┐

                                        SELECT

                 ___|_|_|_|_|_
FEEDBACK ——————————————
                        +
                 __|_||_|___

FIGURE 13

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22
↓
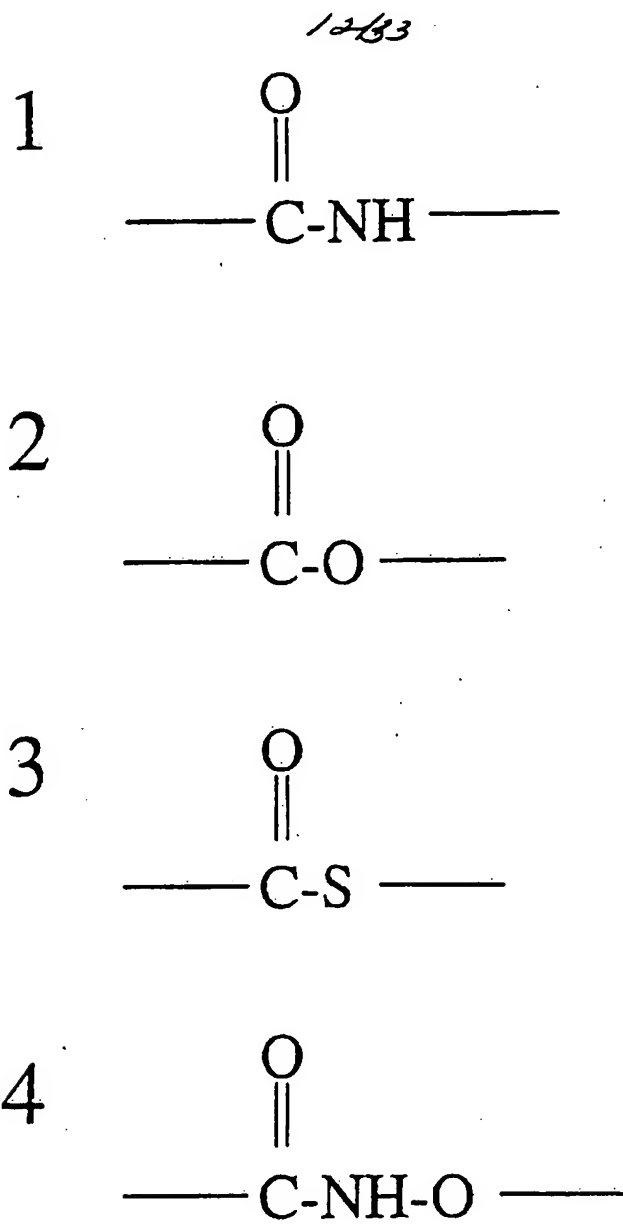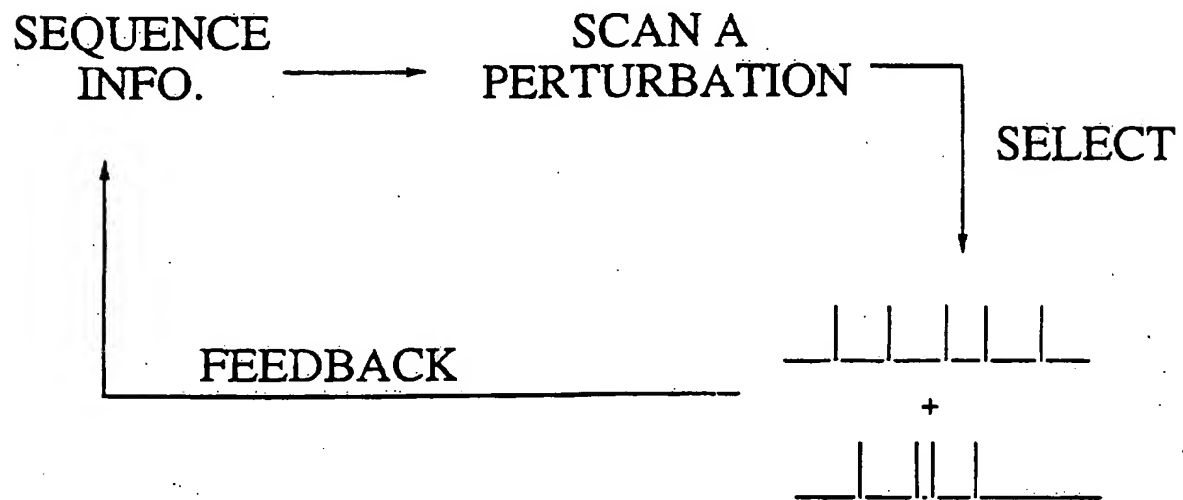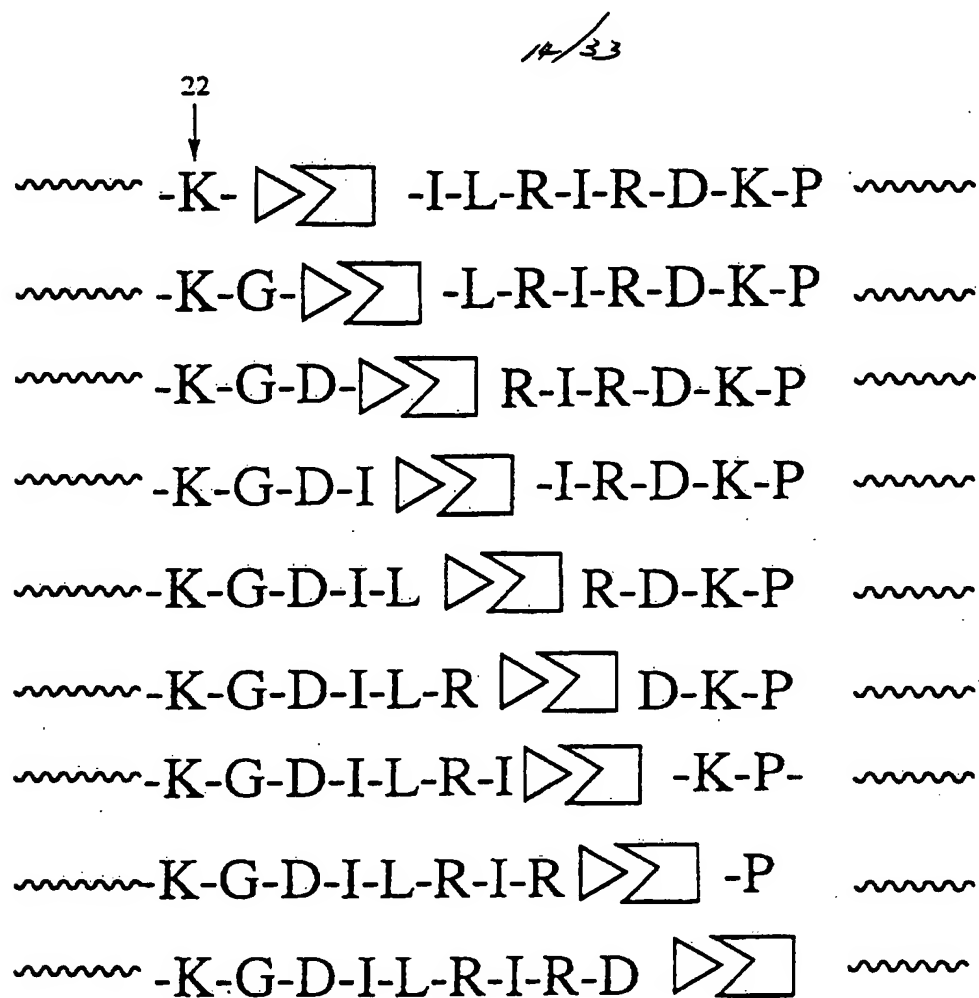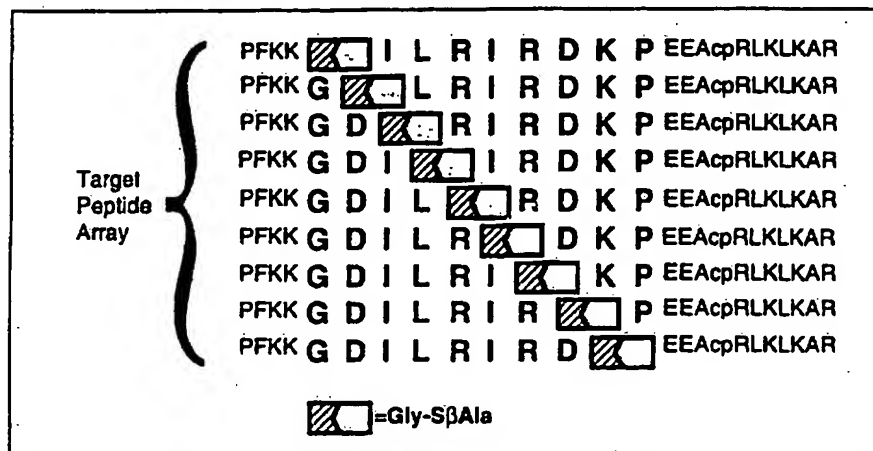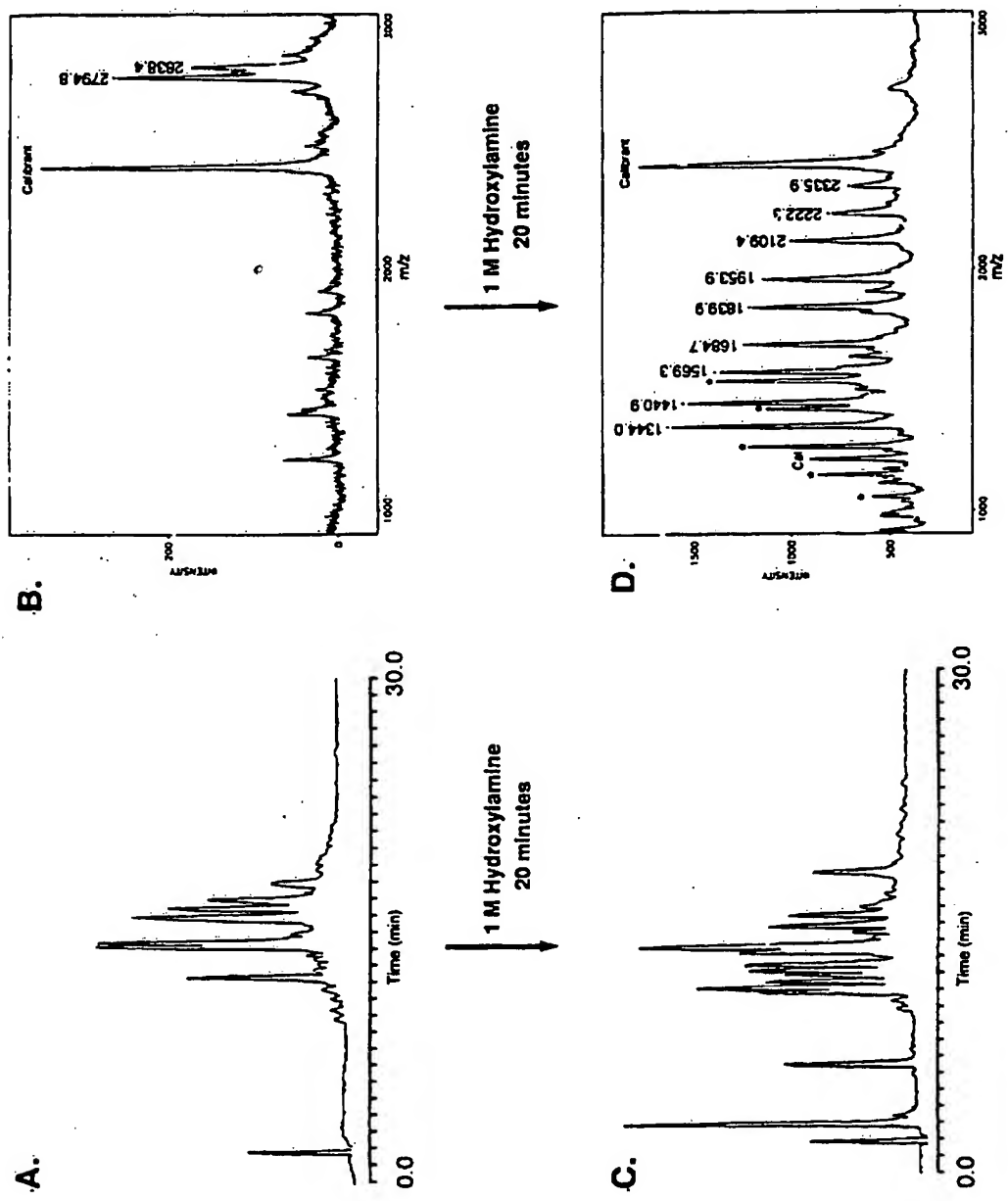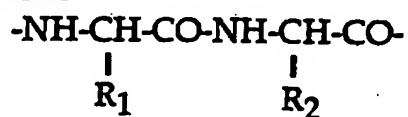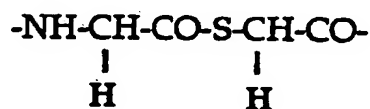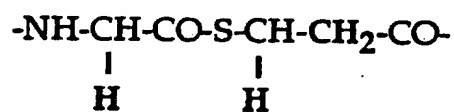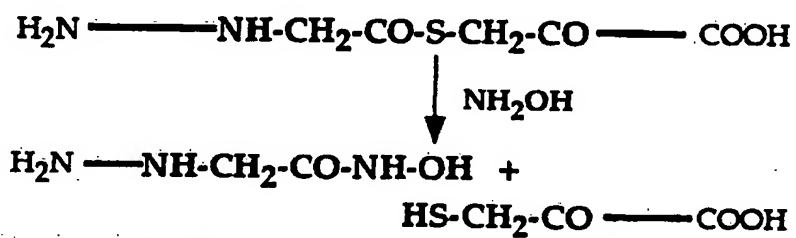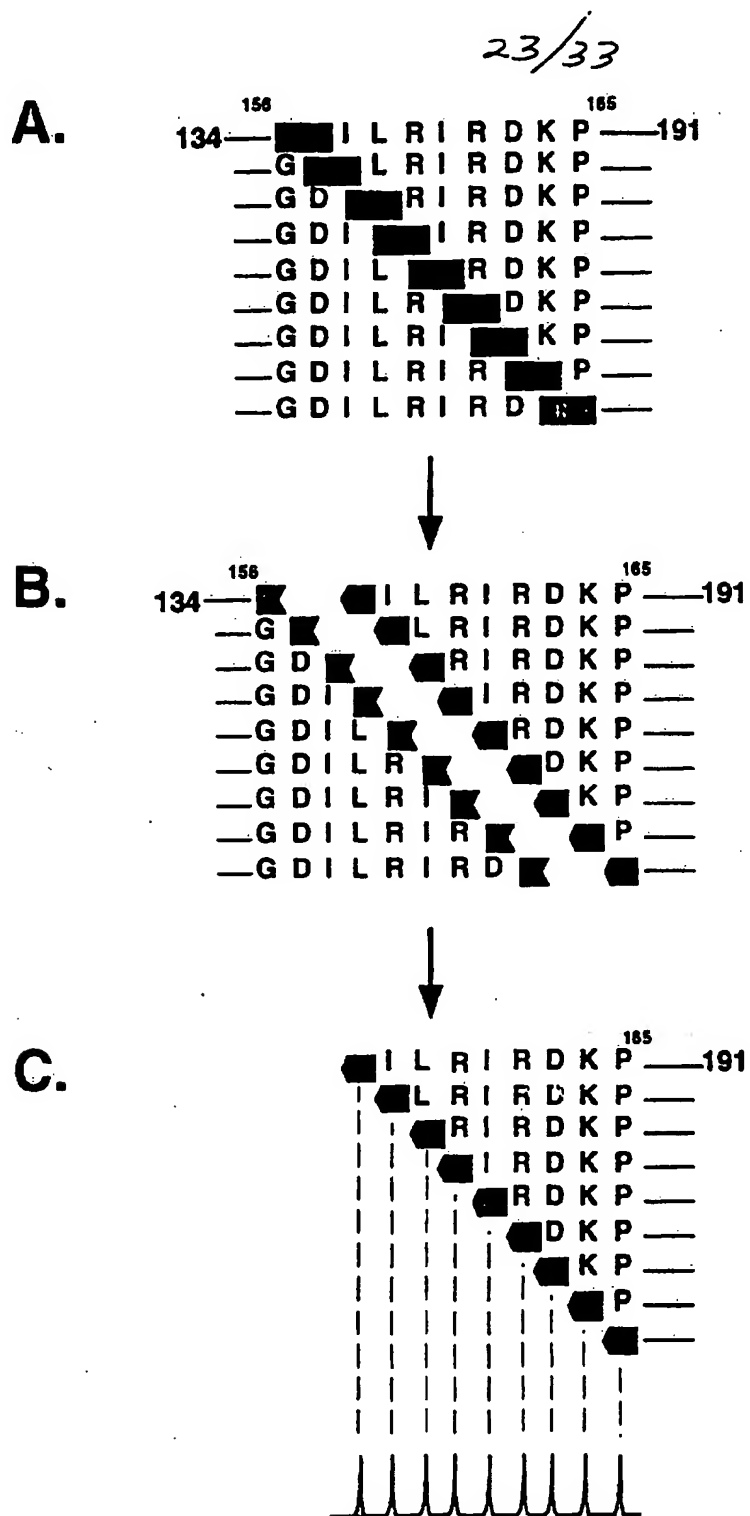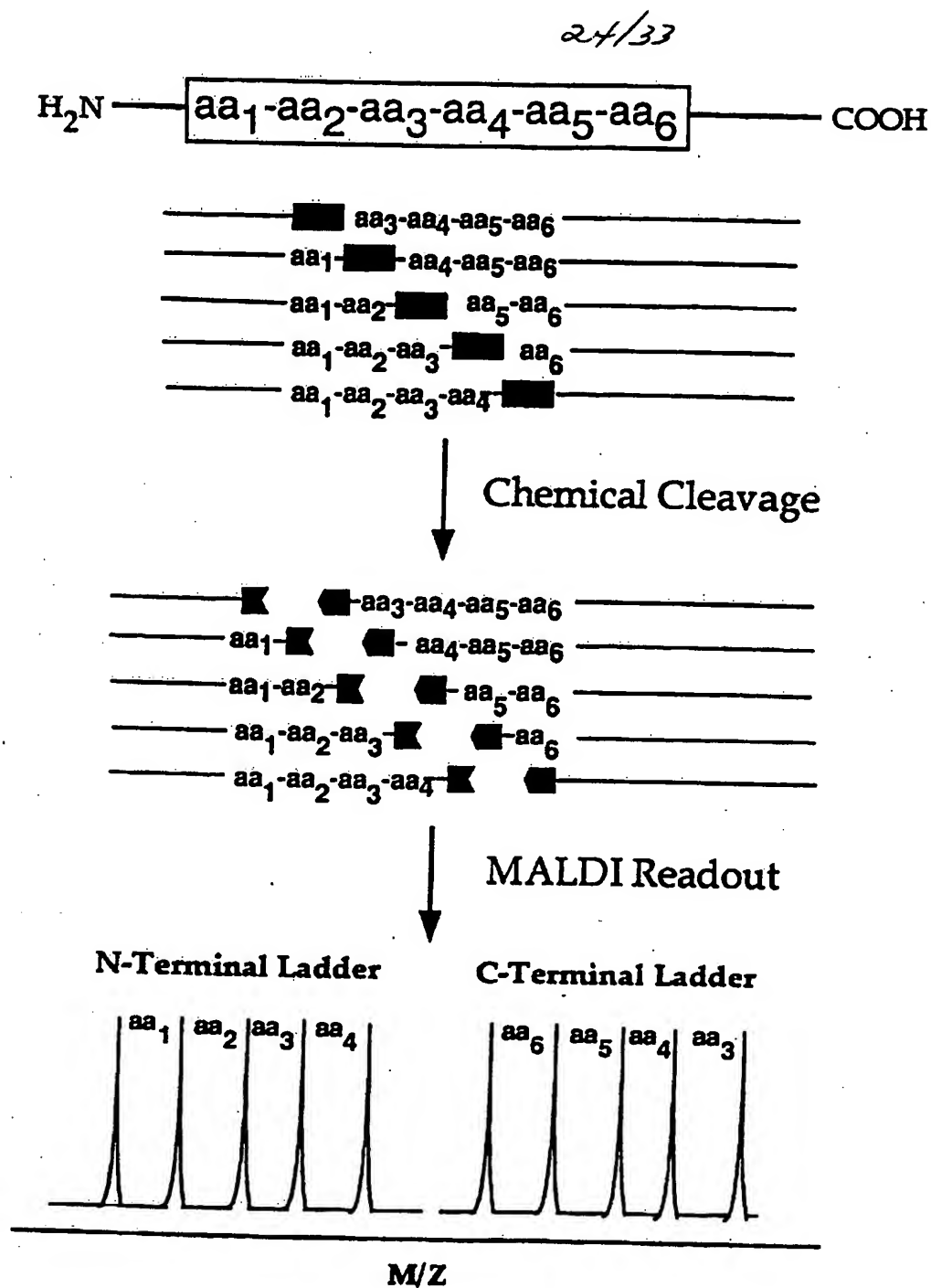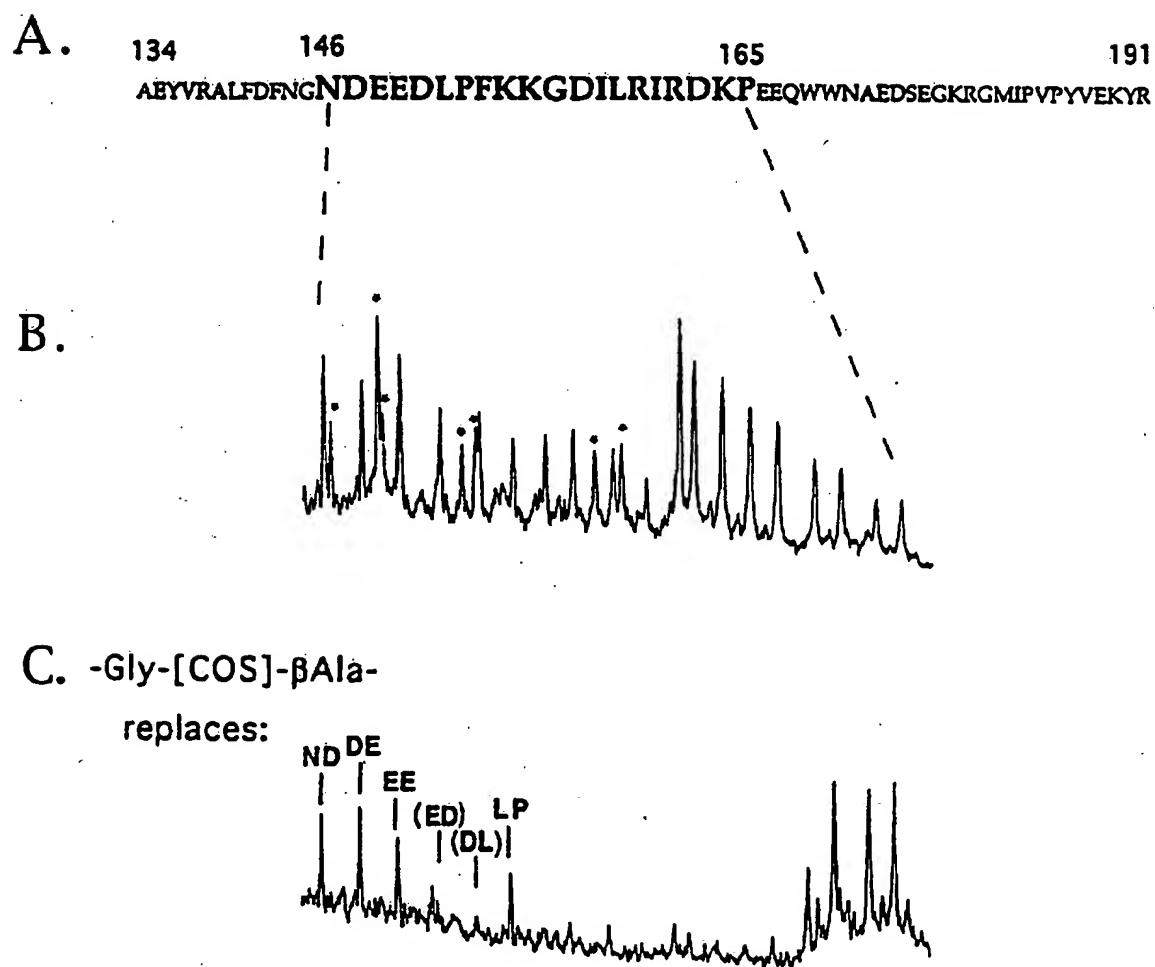
~~~~~~ -K- ▷∑⟩ -I-L-R-I-R-D-K-P ~~~~

~~~~~ -K-G- ▷∑⟩ -L-R-I-R-D-K-P ~~~~

~~~~~ -K-G-D- ▷∑⟩ R-I-R-D-K-P ~~~~

~~~~~ -K-G-D-I ▷∑⟩ -I-R-D-K-P ~~~~

~~~~~ -K-G-D-I-L ▷∑⟩ R-D-K-P ~~~~

~~~~~ -K-G-D-I-L-R ▷∑⟩ D-K-P ~~~~

~~~~~ -K-G-D-I-L-R-I ▷∑⟩ -K-P- ~~~~

~~~~~ -K-G-D-I-L-R-I-R ▷∑⟩ -P ~~~~

~~~~~ -K-G-D-I-L-R-I-R-D ▷∑⟩ ~~~~

FIGURE 14

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A.

Parent peptide sequence: PFKK-**[GDILRIRDKP]**-EEAcpRLKLKAR

B.

Cycle#	A to 1	Neutralize 1	Couple AA_n A , B	+BocGly- SβAla to 1	DMF Wash A , B , 1'	1' to B	+TFA A , B	1 to 1'	DMF Wash A , B
1) Pro[165]	10mL -1 mL	√	√	√	√		√	√	√
2) Lys[164]	9mL -1 mL	√	√ √	√	√ √	√	√ √	√	√ √
3) Asp[163]	8mL -1 mL	√	√ √	√	√ √ √	√	√ √	√	√ √
etc.									
9) Asp[157]	2mL -1 mL	√	√ √	√	√ √ √	√	√ √	√	√ √
10) Gly[156]			√ √		√ √ √	√	√ √		√ √

FIGURE 15

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FIGURE 16

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FIGURE 17

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A.

Array of
Peptide
Analogues

PFKK ▨▢ I L R I R D K P EEAcpRLKLKAR
PFKK G ▨▢ L R I R D K P EEAcpRLKLKAR
PFKK G D ▨▢ R I R D K P EEAcpRLKLKAR
PFKK G D I ▨▢ I R D K P EEAcpRLKLKAR
PFKK G D I L ▨▢ R D K P EEAcpRLKLKAR
PFKK G D I L R ▨▢ D K P EEAcpRLKLKAR
PFKK G D I L R I ▨▢ K P EEAcpRLKLKAR
PFKK G D I L R I R ▨▢ P EEAcpRLKLKAR
PFKK G D I L R I R D ▨▢ EEAcpRLKLKAR

Chemical
Cleavage

B.

Cleaved
Array of
Peptide
Analogues

PFKK ▨   ▢ I L R I R D K P EEAcpRLKLKAR
PFKK G ▨   ▢ L R I R D K P EEAcpRLKLKAR
PFKK G D ▨   ▢ R I R D K P EEAcpRLKLKAR
PFKK G D I ▨   ▢ I R D K P EEAcpRLKLKAR
PFKK G D I L ▨   ▢ R D K P EEAcpRLKLKAR
PFKK G D I L R ▨   ▢ D K P EEAcpRLKLKAR
PFKK G D I L R I ▨   ▢ K P EEAcpRLKLKAR
PFKK G D I L R I R ▨   ▢ P EEAcpRLKLKAR
PFKK G D I L R I R D ▨   ▢ EEAcpRLKLKAR

FIGURE 18

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FIGURE 19

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FIGURE 20

Protein Analogue Array

Readout

Functional
Selection

Inactive
Pool

Active
Pool

Readout

FIGURE 21

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A.

Native Dipeptide:

-NH-CH-CO-NH-CH-CO-
    | |
   R_1 R_2

Gly-S-Gly:

-NH-CH-CO-S-CH-CO-
    | |
   H   H

Gly-S-βAla:

-NH-CH-CO-S-CH-CH$_2$-CO-
    | |
   H   H

B.

Chemical Cleavage:

H_2N ————NH-CH$_2$-CO-S-CH$_2$-CO ———— COOH

                     ↓ NH$_2$OH

H_2N ——NH-CH$_2$-CO-NH-OH +

                 HS-CH$_2$-CO ————COOH

FIGURE 22

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FIGURE 23

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FIGURE 24

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A. 134   146   165   191

ABYVRALFDFNGNDEEDLPFKKGDILRIRDKPEEQWWNAEDSEGKRGMIPVPYVEKYR

B.

C. -Gly-[COS]-βAla-

replaces:

FIGURE 25

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FIGURE 26

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134   156   165   191
AEYVRALFDFNGNDEEDLPFKK**GDILRIRDKP**EEQWWNAEDSEGKRGMIPVPYVEKYR

*analogue
unit
replaces:*

A.-Gly-[COS]-Gly-

(Binding pool)

B.-Gly-[COS]-βAla-

(Binding pool)

FIGURE 27

FIGURE 28

FIGURE 29

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FIGURE 30

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FIGURE 31

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FIGURE 32

FIGURE 33

INTERNATIONAL SEARCH REPORT

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6   C07K1/04

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6   C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category°	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO 95 16209 A (CIBA-GEIGY) 15 June 1995 see the whole document ---	1-16
A	WO 95 11998 A (UNITED BIOMEDICAL INC.) 4 May 1995 see the whole document ---	1-16
T	WO 96 22067 A (UNITED BIOMEDICAL, INC.) 25 July 1996 see the whole document --- -/--	1

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

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Date of the actual completion of the international search	Date of mailing of the international search report
11 February 1997	26. 02. 97

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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category°	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
T	BIOLOGICAL ABSTRACTS, vol. 103, no. 1, 1997 Philadelphia, PA, US; abstract no. 1173, T W MUIR ET AL.: "Probing the chemical basis of binding activity in an SH3 domain by protein signature analysis" XP002025005 see abstract & CHEMISTRY AND BIOLOGY, vol. 3 , no. 10, October 1996, LONDON, pages 817-825,	1-16

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Internal Application No

PCT/US 96/15516

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